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An analysis of resistance to miltefosine and paromomycin in *Leishmania donovani*

A thesis presented by

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This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

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Abstract

Visceral leishmaniasis (VL) is estimated to cause over 300,000 deaths annually, with over 90% of these cases attributed to infection by *Leishmania donovani*. It is therefore a significant global health problem and a major problem in endemic areas such as Nepal and northern India. Drug resistance in the parasite population is reducing the efficacy of clinical drugs, especially antimonials, and new methods to use existing drugs more carefully or develop new drugs are required.

In this study, promastigotes of three Nepalese clinical isolates with different inherent susceptibilities to antimony (Sb) based drugs (i.e. sensitive, Sb-S; intermediate, Sb-I; or resistant, Sb-R) were grown in increasing concentrations of drugs until they expressed tolerance to 74 μM MIL (termed MIL-R) or 97 μM PMM (termed PMM-R). The drug resistant parents were then cloned to assess the impact of resistance to miltefosine (MIL) or paromomycin (PMM) on the 'fitness' and metabolomics/lipidomic profile, thereby elucidating the mechanism(s) responsible for drug resistance and potentially the development of screening assays for MIL or PMM resistance. MIL selected resistance gave rise to significant changes in the lipidome ($p < 0.05$), particularly in lipids involved in the Kennedy pathway, responsible for the biosynthesis of phosphatidylcholines (PCs) and phosphatidylethanolamines (PEs). Importantly there were no mutual alterations in the MIL-R lipidomes. MIL resistance in Sb-S parasites was associated with a significant upregulation of PCs ($p < 0.05$) whereas Sb-R MIL-R clones appeared to have significant depletions in their PC content ($P < 0.05$) likely as a consequence of

maintaining their PE content. MIL uptake in Sb-S and Sb-R MIL-R clones was significantly reduced compared to their corresponding wild-type (WT, $p < 0.05$) and MIL resistance was associated with disruptions in the gene encoding the *Leishmania donovani* miltefosine transporter (LdMT) protein in both MIL-R clones. PMM resistance had significantly higher amounts of proline, glutamine, methionine and serine, which have all been associated with increased resistance to oxidative stress. MIL-R clones were significantly more susceptible to killing by superoxide ($p < 0.05$) and NO induced killing compared to their corresponding WT strain and were significantly less infective ($p < 0.05$) to macrophages. In contrast, PMM-R was associated with an increase in parasite 'fitness' as PMM-R promastigotes and intracellular amastigotes were more resistant to NO ($p < 0.05$) and killing by interferon gamma/liposaccharide stimulated macrophage killing compared to their WT parents ($p < 0.05$). However the effects of MIL resistance or PMM resistance were strain dependant indicating that there was no simple assay to identify drug resistant strains.

Additionally, a library of novel minor groove binding (MGB) molecules, developed at the University of Strathclyde, were screened for their potential as new antileishmanial drugs. Twelve out of twenty-nine compounds tested had inhibitory effects on *L. donovani* in macrophage screening studies, causing > 75% suppression in parasite burdens compared to controls. One compound, MGB-3-NO, was tested in a murine model of VL. Treatment with MGB-3-NO (50 mg/kg) solution had no antileishmanial activity but treatment with MGB-3-NO encapsulated into non-ionic

surfactant vesicles to increase its targeting to macrophages, caused a significant reduction in liver parasite burdens compared to controls ($p < 0.05$).

The results of this study identified similar types of metabolic and lipidomic changes in MIL-R and PMM-R parasites compared to WTs but the changes were clone-specific indicating that there was no clear and predictable mechanism to either MIL or PMM resistance selected in promastigotes.

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Table of Contents

List of Figures

List of Tables

Abbreviations

°C	Degree Celsius
AmB	Amphotericin B
APC	Antigen presenting cell
bp	Base pair
BLI	Bioluminescence
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CDP	Cytidine diphosphate
CK	Choline kinase
CL	Cutaneous Leishmaniasis
CT	Choline phosphotransferase
CR3	Complement receptor 3
CPCT	Choline-phosphate cytidylyltransferase
DALY	Disability-adjusted life years
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EK	Ethanolamine kinase

ELISA	Enzyme-linked immunosorbent assay
EPCT	Ethanolamine-phosphate cytidylyltransferase
ET	Ethanolamine phosphotransferase
FCS	Foetal calf serum
GP63	Glycoprotein 63
GSH	Glutathione
HRP	Horseradish peroxidase
IC_{50}	Half maximal inhibitory concentration
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
ISC	Indian sub-continent
IVIS	*In vivo* imaging system
kDa	Kilo Daltons
L-AmB	Liposomal amphotericin B
LCMS	Liquid chromatography mass spectrometry
LdMT	*Leishmania donovani* miltefosine transporter
LPC	Lysophosphatidylcholines
LPS	Lipophosphoglycan
MIL	Miltefosine
ML	Mucosal Leishmaniasis

MHC	Major Histocompatibility complex
mRNA	Messenger RNA
MSF	Médecins sans Frontières
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NO	Nitric oxide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PENM	Phosphatidylethanolamine N-methyltransferase
PI	Phosphatidylinositol
PL	Phospholipid
PS	Phosphatidylserine
PKDL	Post kala-azar dermal Leishmaniasis
PMM	Paromomycin
PPG	Proteophosphoglycans
PSG	Promastigote secretory gel
PV	Parasitophorous vacuole
RNA	Ribonucleic acid
ROS	Reactive oxygen species

RNS	Reactive nitrogen species
Sb	Antimony
Sb^{III}	Trivalent antimony
Sb^{V}	Pentavalent antimony
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SD	Standard deviation
SE	Standard error
SSG	Sodium stibogluconate
SL	Sphingolipid
SM	Sphingomyelin
Th	T-helper cells
TNF-α	Tumour necrosis factor-α
T reg	T regulatory
TSH	Trypanothione
VL	Visceral Leishmaniasis
WHO	World Health Organisation

Chapter 1. Introduction to Leishmaniasis

1.1 Leishmaniasis

Leishmaniasis is a spectrum of disease caused by infection with the protozoan parasite of the genus *Leishmania*. It is thought that *Leishmania* parasites may have evolved as long as 50 million years ago, at the same time as the evolution of mammals was beginning. DNA evidence for human infections with *Leishmania* has been traced back as far as 4,000 years in Egyptian mummies (Tuon *et al.*, 2008). The first scientific recording of *Leishmania* was in 1885 when Cunningham noted the organisms while examining skin lesions of patients in India. At the time however, he incorrectly identified the parasites as a species of fungus (Cunningham, 1885). It wasn't until 1898 that Borovsky correctly identified the same organism as a protozoan when examining skin lesions of patients, this time in Turkmenistan. Due to the obscurity of the Russian military journal that his findings were published in, his work was not recognised at the time (Hoare, 1938). It was instead a Glaswegian medic serving in the British army in India, William Leishman, who reported to the British Medical Journal the presence of intracellular parasites in the livers of Indian patients who had succumbed to the disease kala-azar (Leishman, 1903). In the same year, Charles Donovan, an Irish doctor, reported similar findings from liver smears taken from Indian patients (Donovan, 1994). As recognition of their discoveries the parasite was classified as *Leishmania donovani*.

Approximately 20 different species of *Leishmania* are known to cause disease in humans. The vector borne parasite is transmitted by sandflies, small biting insects that feed on the blood of mammals. As with the *Anopheles* mosquito and the malaria causing *Plasmodium* parasite, only female sandflies transmit *Leishmania* as

males do not require a blood meal. There are four classifications of leishmaniasis based on clinical symptoms; mucosal leishmaniasis (ML), cutaneous leishmaniasis (CL), visceral leishmaniasis (VL, also known as kala-azar) and post kala-azar dermal leishmaniasis (PKDL). Of the four forms of the disease, CL is the most common, while VL is most serious and fatal if left untreated. Symptoms of VL include intermittent fever, hepatosplenomegaly (enlarged liver and spleen), enlarged lymph nodes, significant weight loss and anaemia (Chappuis *et al.*, 2007). Two species of *Leishmania* are responsible for VL; *L. donovani* and *L. infantum.* The causative species depends on both the geographical location of infection and age of the patient. *L. donovani* is found in the Old World (Eastern Hemisphere) and infects people of all age groups. In contrast, *L. infantum*/*L. chagasi* is New World (Western Hemisphere) and predominantly infects children and the immuno-suppressed.

Sandflies of the species *Phleobotomus* are responsible for the transmission of Old World *Leishmania*. In the New World, it is sandflies of the genus Lutzomyia that act as vectors for the parasite. With respect to *Phlebotomine* sandflies, there are known to be roughly 30 species capable of transmitting the parasites (Pearson and Sousa, 1996).

Most recent World Health Organisation (WHO) figures indicate that 350 million people, living in 98 countries are at risk of contracting a form of leishmaniasis, resulting in between 1-2 million cases annually. With VL, there are an estimated 300,000 cases annually leading to in excess of 40,000 deaths (Nagle et al., 2014). These figures are likely to be underestimating the disease incidence as in VL

endemic areas, the disease is often misdiagnosed or not reported, and exact causes of deaths are not always recorded (Collin *et al.*, 2006, Singh *et al.*, 2006a). Aside from the high numbers of mortality caused by the parasite, leishmaniasis accounts for 2,357,000 Disability-adjusted life years (DALYs) lost due to ill effects caused by the disease. This figure is also likely to be an underestimate as not only is data hard to collate, the data does not account for secondary infections such as PKDL which would also be significant (WHO, 2010). Visceral Leishmaniasis is found in 88 countries throughout the world, even encroaching in to southern Europe along the Mediterranean (Figure 1.1). The majority of infections however occur in India, Nepal, Bangladesh, Sudan and Ethiopia where upwards of 90% of all VL infections are found. It is no coincidence that these aforementioned countries are ranked as some of the poorest in the world (Boelaert *et al.*). In India alone, an estimated 100,000 cases occur annually, with 90% of these cases found in the Bihar region of North-East India (Bora, 1999).

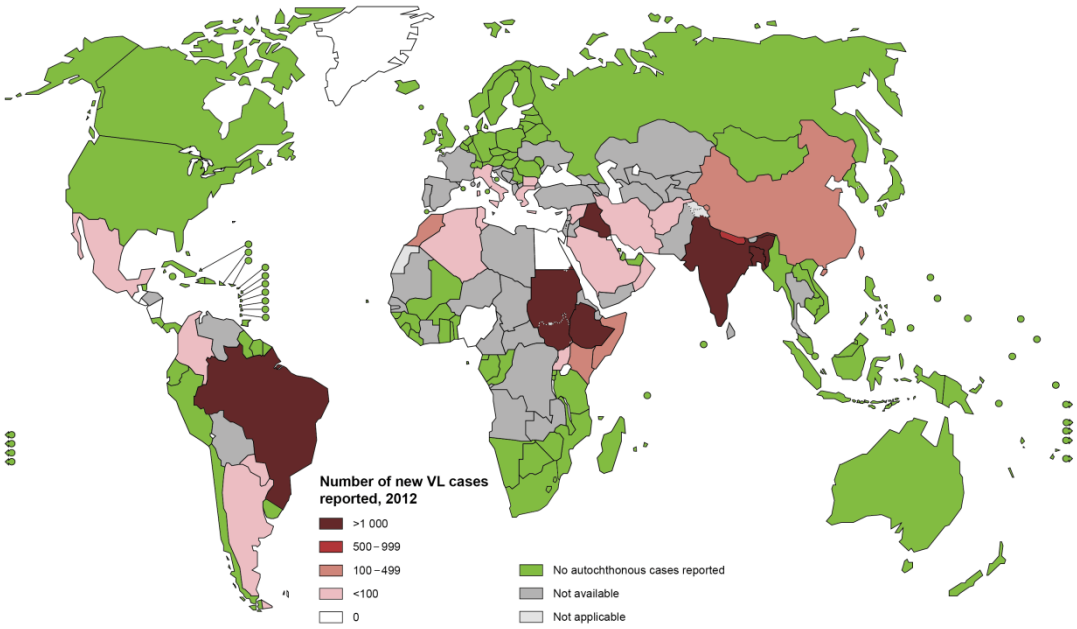


Figure 1.1. Global distribution of areas endemic to Visceral Leishmaniasis. Figure adapted from WHO website found at http://www.who.int/leishmaniasis/en/.

1.2 Lifecycle

Leishmania has two distinct morphological forms in its lifecycle; the amastigote that resides inside mammalian host macrophages and the promastigote stage found predominantly in the midgut of the sandfly (Figure 1.2). Amastigotes differ from promastigotes by having a rounded shape and a flagellum that is confined to the flagellar pocket. Promastigotes on the other hand are motile as result of their more prominent flagellum and elongated bodies. Three species of Phlebotomine sandflies act as vectors for L. donovani and humans throughout the Indian subcontinent and East Africa; *P. argentipes, P. orientalis,* and *P. martini*.

Sandflies do not feed in the same manner as most biting insects such as *Anopheles* mosquitoes by injecting a proboscis into host's capillaries and sucking blood. Instead, sandflies inject their mouthparts into its victim's skin and slice it, causing a superficial wound in order to feed from the resulting blood pool. *Leishmania* promastigotes are regurgitated from the sandfly gut into the pool of blood during feeding and enter the body of the mammalian host through the open wound. Promastigotes are phagocytosed by macrophages and become enveloped into a phagosome. Once internalised, enzyme containing lysosomes fuse with the phagosome, creating the highly acidic phagolysosome, which in essence becomes a parasitophorous vacuole (PV). The different species of *Leishmania* form varied sizes of PV inside their host macrophage, with *L .donovani* known to produce a small, tightly formed PV. Here, the promastigote loses its flagella and transforms into the rounded amastigote inside the PV where it will multiply via asexual replication. The resulting amastigotes are released from the macrophage, generally thought to be

via bursting of the infective macrophage, to invade nearby macrophages and continue the replication cycle or until the macrophage is up taken in another sandfly blood meal.

Interaction and avoidance of *Leishmania* with the host immune system is mediated through a number of cell surface molecules. The cell surface of promastigotes and amastigotes are coated in a variety of stage specific proteins that mediate how the two stages interact with the mammalian host, for example lipophosphoglycan (LPG) is unique to promastigotes. Interactions with macrophages and amastigotes are instead thought to be facilitated mainly by proteophosphoglycans (PPG) on the amastigote and IgG of the host (Ilgoutz and McConville, 2001, Peters *et al.*, 1995). The amastigote parasite is reliant on its host cell, usually a macrophage, for transport around the body, where it localises in the main sites of infection; the spleen, liver and bone marrow. Access to such areas is ideal from a parasite's perspective as they are macrophage rich environments with a supply of new macrophages to infect. While amastigotes residing in these sites are important in maintaining an infection, it is only amastigotes present in macrophages in the peripheral blood that will be able to be transmitted back into a sandfly when it takes its next blood meal (Bates, 2007).

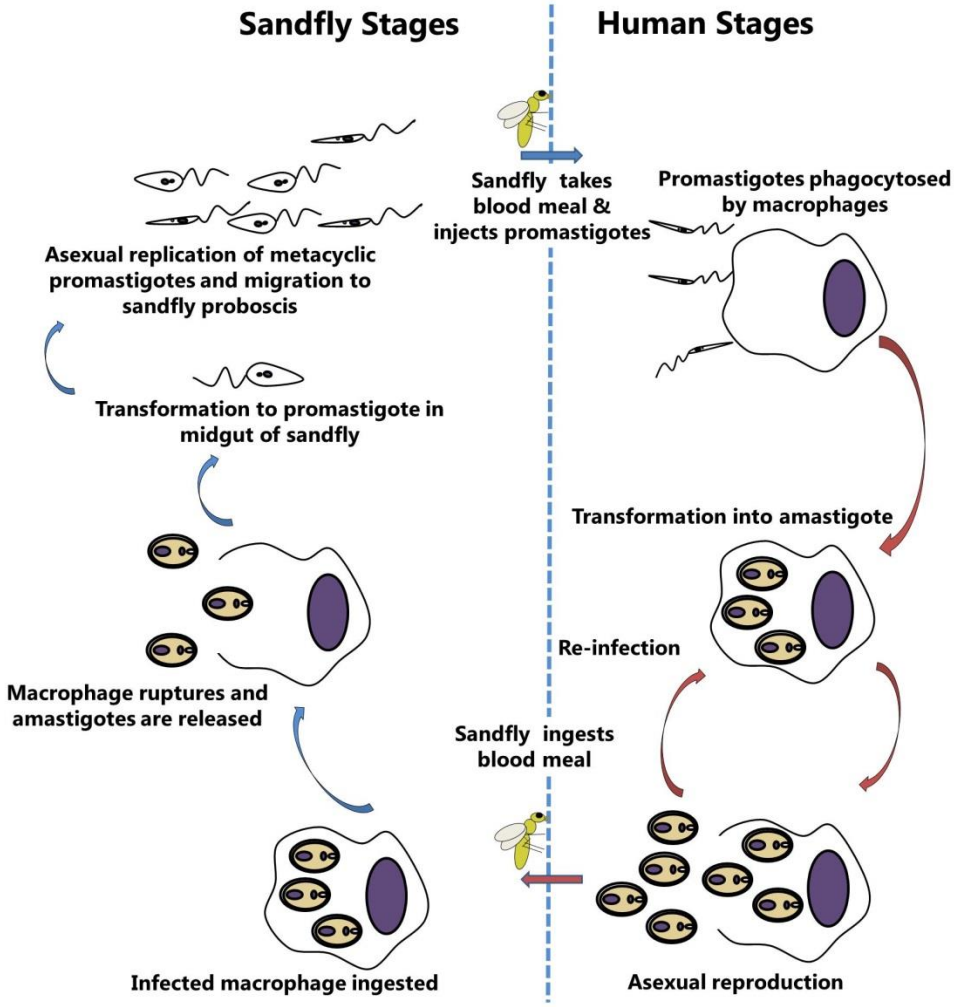


Figure 1.2. Lifecycle of *Leishmania* through human host and sandfly vector.

Once inside the sandfly, the high midgut pH coupled with a drop in environmental temperature triggers the amastigote's transformation into its motile, promastigote form (Bates and Rogers, 2004). Promastigotes go through four distinct stages inside a sandfly. The first of these is the procyclic promastigote which is similar in size to the amastigote with an anterior flagellum. This replicative stage remains in the midgut blood meal of the sandfly dividing asexually over a period of 1-2 days. After this time both the main body and flagellum elongate developing in to a nectomonad promastigote. This form is highly mobile and responsible for the migration of the parasite out of the blood meal and towards the anterior end of the sandfly (Stierhof *et al.*, 1999). Migration of the nectomonad form continues until it reaches the boundary between the midgut and foregut, marked by the stomodeal valve (Sacks and Kamhawi, 2001). Once here, the nectomonad shortens to become the replicative leptomonad (Gossage *et al.*, 2003). Leptomonad parasites produce a substance termed promastigote secretory gel (PSG), a glycoprotein that plays a critical role in the transmission of the promastigote into its next mammalian host. It causes obstruction in the sandfly midgut that it must regurgitate and expel. This allows metacyclic promastigotes to exit the sandfly gut and onto the skin of a human [reviewed in (Bates, 2007)]. Eventually leptomonads become metacyclic promastigotes, characterised by their short body and elongated flagellum. It is this stage of the promastigote that is responsible for transmission back into a mammal (Rogers *et al.*, 2002). The lifecycle is continued when metacyclics re-invade a human host during the infected sandflies next blood meal.

1.3 Immunity to *Leishmania*

Unlike the majority of other pathogens that rely on evading its host immune system to proliferate, an interaction with host macrophages is essential for *Leishmania* to survive within its human host. Immunity to *Leishmania* is activated in response to the initial sand fly bite, which induces a local inflammatory response, recruiting neutrophils and monocytes to the infection site (Mougneau *et al.*, 2011).Host complement fragment C3 is activated by the presence of foreign promastigotes and binds to complement receptor 3 (CR3) on macrophages via C3bi. Metacyclic promastigotes are coated in various phosphoglycans, such as LPG and PPG that interact and manipulate the complement pathway to aid parasite survival. LPG prevents the C5-C9 membrane attack complex forming and helps prevent complement mediated lysis occurring. Various protein kinases on the surface of metacyclics phosphorylate the complement components C3, C5 and C9 which also help to deactivate the complement pathway. Another parasite surface molecule glycoprotein 63 (GP63) cleaves C3b to produce the inactive form C3bi and prevents the formation of C5 convertase. C3bi also facilitates the uptake of promastigotes in to the macrophage via CR3 thus avoiding binding to CR1 which would lead to IL-12 production and parasite clearance. This ability to circumvent CR1 binding and subsequent inhibition of IL-12 macrophage activation is termed the “silent entry” of the parasite.

Once inside its macrophage host the promastigote must transform in to the amastigote stage which is more suited to life inside the phagolysosome. To do this, it must prolong the life of the macrophage host by preventing apoptosis. *L.*

donovani has been shown to do this through the secretion of TNF-α and by averting the apoptotic caspase cascade induced via oxidative burst (Srivastav *et al.*, 2014). Control of *Leishmania* infection is largely governed by the phenotype of the T-helper (Th) cell response generated and via the production of the appropriate cytokines to stimulate parasite killing within the host macrophage. While it is not clear cut, in general it is considered that a Th1 response mediated initially via IL-12 production by dendritic cells/macrophages is associated with clearance of the *Leishmania* infection. On the other hand, a Th2 mediated response to VL by IL-10 and TGF-β is associated with a susceptibility and progression of the disease. This is perhaps an over simplified view however, drawn primarily from work using murine models. In human VL infections, the Th response phenotype is not as black and white and involves a mixed response of several cytokines (Cecilio *et al.*, 2014). IL-12, produced by antigen presenting cells (APCs) such as macrophages and dendritic cells is one of the most important cytokines in driving the immune response to *L. donovani*. In essence, the parasite's ability to prevent IL-12 production plays a significant role in parasite survival and exacerbation of the disease. Parasite degradation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) via cysteine proteases has been linked to an impairment of IL-12 production and correlated with parasite proliferation. Another important role of IL-12 is in priming T cells maturation into Th1 cells, which in turn produce IFN-γ. Both TNF-α and IFN-γ are important in stimulating macrophage killing mechanisms via the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). As with IL-12, interference with host production of IFN-γ is associated with parasite survival. *L.*

donovani are known to inhibit IFN-γ production via a disruption of phosphorylation of the IFN-γR-α subunit which in turn disrupts expression of IFN-γ. Both IL-12 and IFN-γ help promote the formation of hepatic granulomas in the liver. Granulomas are organised structures formed from monocytes, Kupffer cells, $CD8^+$ and $CD4^+$ T cells. Inside granulomas parasites are starved of nutrients and oxygen and exposed to high local concentrations of ROS and RNS, leading to parasite death (Fig. 1.3).

While IL-12 production is generally considered detrimental to *L. donovani* survival, IL-10 production is associated with infection and persistence of the parasite. Knock out mice, deficient in IL-10 were shown to be resistant to infection with *L. donovani* (Murphy *et al.*, 2001). In active VL infections, infected macrophages produce IL-10 instead of IL-12 and antigenic stimulation results in the production of parasite-specific $CD4^+$ and $CD25^+$ T-reg cells, resulting in the production of even more IL-10 and stimulation of a Th2 immune response. $CD25^-Foxp3^-$ T cells have also been implicated in the production of IL-10 and pathogenesis of *L. donovani.* IL-10 is considered to be a homeostatic cytokine and is involved in preventing tissue damage. It is also capable of suppressing the production of pro-inflammatory molecules such as ROS, TNF-α, IL-12 and RNS that directly downregulate host protective immune responses against *L. donovani.* This allows the parasite to proliferate and persist within the body.

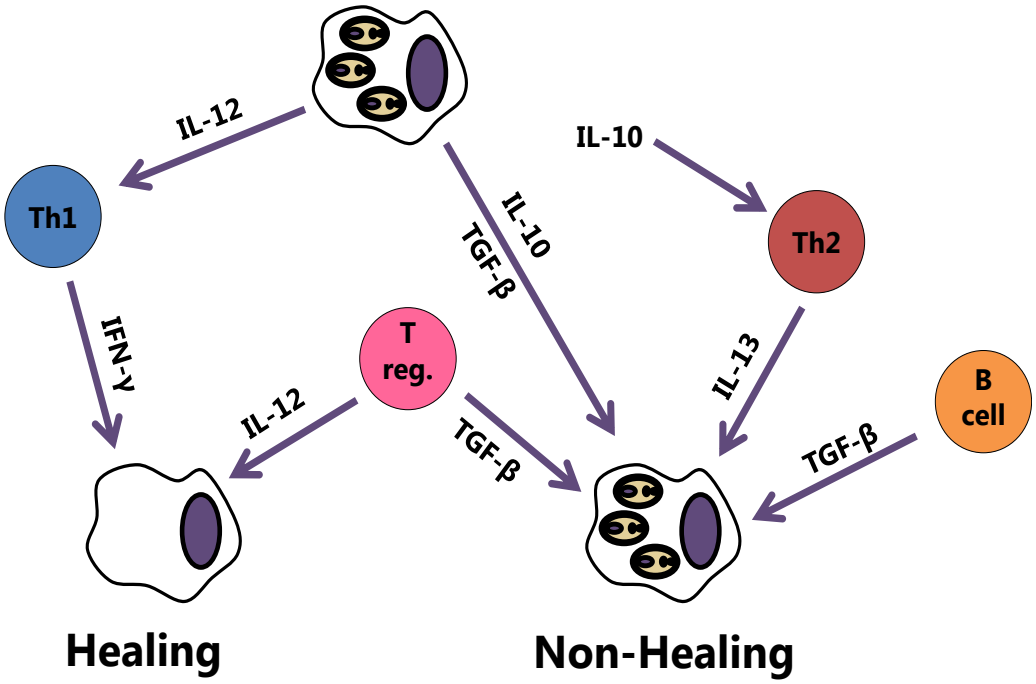


Figure 1.3. Immune responses to *Leishmania* and the cytokines responsible for driving healing and non-healing infections.

L. donovani is capable of decreasing macrophage expression of MHC I and MHC II molecules as well as impairing MHC II binding with T cell receptors and thus decrease antigen presentation via APCs. Infection with *L. donovani* has also been correlated with a reduction of $CD8^+$ and $CD4^+$expression on the surface of T helper cells. The exact role of B cells in VL is however less well understood but active infection is associated with high titres of non-protective antibody (Gupta *et al.*, 2013). Polyclonal antibodies then appear at approximately day 7 post infection and B cell numbers in draining lymph nodes are markedly increased over the course of infection. B cell deficient mice are generally more resistant to VL and passive transfer of IgM or IgG into these deficient mice results in increased susceptibility (Deak *et al.*, 2010). This suggests that cell-mediated immunity is more important than humoral immunity in mediating protection.

1.4 Drug Treatment of leishmaniasis

Current options to treat VL are limited with only four anti-leishmanial compounds routinely used to treat the disease. Each of the available chemotherapy options come with its own significant disadvantage associated with its use, including high cost, toxicity or administration difficulties. Combined with the threat of drug resistance and the small number of novel compounds in the drug discovery pipeline, the current situation concerning the treatment of VL is not encouraging. The drugs currently in the pipeline will be briefly mentioned in Chapter 8.

1.4.1 Pentavalent Antimonials

Pentavalent Antimonial compounds (Sb^{V}), sodium stibogluconate (SSG, Figure 1.4) and meglumine antimoniate based on elemental antinomy (Sb) have been used to treat leishmaniasis since the 1930s. In the majority of endemic countries, with the exception of the Indian sub-continent (ISC), SSG is regarded as the primary treatment option against VL. At $10 per treatment (Table 1.1), it is one of the cheapest therapeutic options, making it the preferential treatment option in poorer countries. SSG must be administered via parenteral routes such as intramuscular or intralesional injection. The recommended course of treatment is a daily 20 mg Sb^{V}/kg injection for 28-30 days (WHO, 2010). A number of other side effects are associated with SSG treatment such as pancreatitis and arrhythmia. Despite the success of SSG, treatment failure due to resistance is now a major concern in India and Nepal where the drug is effectively obsolete in particular regions due to acquired resistance of the parasite. Clinical advice is now recommending alternatives such as miltefosine (MIL) and amphotericin B (AmB) as the first line treatments of the disease in these areas. Despite being used for nearly 80 years, the exact mode of action of antimonial compounds is not fully understood. Antimonials are known to function as pro-drugs, requiring the pentavalent antimony (Sb^{V}) to be reduced to the trivalent antimony form (Sb^{III}) in order to become active. The exact location where the reduction from Sb^{V} to Sb^{III} occurs i.e. within the macrophage, parasite or both, has so far remained inconclusive (Haldar *et al.*, 2011). Promastigotes are only susceptible to Sb^{III}, indicating that they are incapable of reducing Sb^{V} to Sb^{III} (Ephros *et al.*1999). This stage-specificity indicates that

amastigotes and/or macrophages have a mechanism capable of reducing Sb^{V} while promastigotes do not.

Antimonials act upon the parasite by targeting both trypanothione (TSH) and glutathione (GSH), fatty acid and carbohydrate metabolism. TSH is a thiol unique to members of the trypanosomatid family of parasites (*Leishmania* and *Trypanosoma spp*.) and has therefore always been an attractive target for drug design. TSH consists of two GSH molecules linked by a spermidine polyamine group (Fairlamb and Cerami, 1992). The primary role of TSH is protecting the parasite from chemical and oxidative stress by maintaining the internal thiol redox balance. The molecule acts an electron donor and can neutralise the effects of free radicals produced by macrophages and neutrophils as part of the host protective immune response i.e. nitric oxide and superoxide (Krauth-Siegel *et al.*, 2003). Sb^{III} effects a rapid efflux of TSH and GSH from the parasite (>90%) while at the same time inhibiting TSH reductase activity thus preventing further TSH production and converting any leftover TSH to its disulphide form. This efflux of TSH and the subsequent accumulation of disulphides creates a lethal imbalance of thiol redox potential within the parasite that leads to cell death (Wyllie *et al.*, 2004).

1.4.2 Amphotericin B

Amphotericin B deoxycholate, originally extracted from *Streptomyces nodosus*

an anti-fungal agent marketed as Fungizone®. Anti-leishmanial activity was shown in the 1950s, however it was not until 1995 that it was used to treat SSG-resistant *Leishmania* cases (Jha *et al.*, 1995, Cappuccino and Stauber, 1959). Despite

possessing high anti-leishmanial activity, the deoxycholate form of AmB is associated with a number of side effects, most notably nephrotoxicity (Clements and Peacock, 1990). To counteract this toxicity, several lipid formulations of AmB were developed during the 1980s. These proved to be as effective as conventional deoxycholate but had the distinct advantage of significantly reduced side effects (Hiemenz and Walsh, 1996, Berman *et al.*, 1998). To date, eight formulations of L-AmB have been approved by the US-FDA (Balasegaram *et al.* 2013). Liposomal-AmB is rapidly concentrated to the liver and spleen which helps to target drug delivery to organs with high a parasite burden while reducing the exposure of free drug to other organs (Sundar *et al.* 2015). Although predominantly used as the second line of defence against VL, AmB has become the drug of choice in areas of high SSG resistance such as Northern India and Nepal. L-AmB is not stable at high temperatures and must be stored < 25°C (AmBisome guidelines). The average temperatures in *Leishmania* endemic regions of India and East Africa far exceed this temperature, and especially in Bihar India, the average temperatures exceed 25°C from February to November (www.worldweatheronline.com/bihar-weather-averages/bihar/in. Last accessed 01/02/2016). For this reason, the logistics of transporting and storing L-AmB at the correct temperature can be problematic.

Unlike SSG, L-AmB must be administered through intravenous injections, although infusion of L-AmB takes significantly longer and is performed over a number of days. At the WHO preferential price of $18 for a 50 mg vial (Table 1.1), L-AmB treatment has reduced in price significantly for patients. However, treatment with L-AmB requires that the patient to remain in hospital for the duration of the regimen. This

in turn increases indirect costs associated with treatment and can be a prohibiting factor, particularly in poorer countries (Sundar and Chakravarty, 2010). In addition, from a socio-economic perspective, if the primary wage earner is hospitalised for 10 days, then considerable financial strain is placed on the family. Encouragingly, a study in India reported a 95% cure rate of VL using only a single-dose of L-AmB at 10 mg/kg (Sundar *et al.*, 2008) showing it to be highly efficacious at treating the parasite. A single-dose treatment is advantageous as it requires only one visit to a hospital and so reduces both overall cost and time spent in bed. Such a strategy of single-dose L-AmB, either on its own or in combination with another anti-leishmanial drug could be an effective way of extending the availability of a highly effective drug by lowering the costs of treatment and making the drug more accessible to patients. Single dose L-AmB has been the treatment of choice in India since 2014 (Sundar *et al.* 2010, WHO Technical Report 2010, Sundar *et al.* 2014). Several studies into the cost effectiveness of combination therapy have been reported and are discussed later. Since the introduction of L-AmB, variation in the dose efficacy in different populations has been reported. For example patients in India and East Africa showed significant differences in the curative dose of L-AmB (Sundar *et al.*, 1997a, Sundar *et al.*, 2002c). As a result, WHO guidelines vary with geographical region, ranging from 10-20 mg/kg total dose over a period of up to 10 days. Furthermore, the different brands of L-AmB have varying degrees of efficacy and safety (Sundar *et al.* 2015). Current WHO guidelines recommend a single dose of AmBisome at 10 mg/kg L-AmB which has previously been shown to achieve a cure rate of > 95% in India (Sundar *et al.* 2010) A recent Phase II trial in India

focusing on an indigenously manufactured formulation, Fungisome, found a single dose of 15 mg/kg body weight could achieve a definitive cure rate of 93% at the six month follow up (Sundar *et al.* 2015). In contrast to the high efficacy of L-AmB in India, treatment failure in East Africa has been found (Kalili *et al.* 2014). In this study, cohorts with patients were treated with either multiple doses (7 times 3 mg/kg on days 1-5, 14, and 21) or single doses (either 7.5 mg/kg or 10 mg/kg) L-AmB. Definitive cure of patients treated with multiple doses was only 85% while single dose treatment of 7.5 and 10 mg/kg achieved only 40% and 58% respectively. This apparent treatment failure with L-AmB in East Africa suggests that although the drug is highly efficacious in India, it may not be suitable for VL treatment in East Africa.

Amphotericin B acts upon multiple *Leishmania spp.* and fungi by interacting with and altering the permeability of their plasma membranes. Specificity between human and pathogen plasma membrane is achieved due to the higher affinity of amphotericin B towards ergosterol, an episterol found in the membranes of *Leishmania* instead of cholesterol, the episterol in human membranes (Saha *et al.*, 1986, Beggs, 1994).

Table 1.1. Drugs currently available for the treatment of visceral leishmaniasis. Calculations are based on information from Bihar, India and factor in cost of hospitalisation. Prices may change depending on the location of treatment. Information adapted from (Nagle et al., 2014).

Drug	Brand Names	Cost ($)	Administration	Side Effects	Resistance in Field
Sodium Stibogluconate	Pentostam	50 - 70	Intramuscular	Vomitting, Nausea, Cardiotoxicity, Pancreatitis	Yes
Liposomal Amphotericin B	AmBisome, Amphotec, Abelcet	280	Intravenous	Nephrotoxicity, Hepatotoxicity	No
Miltefosine	Impavido, Miltex	70*	Oral	Teratogenic, Gastrointestinal	Possibly
Paromomycin	Monomycin	10	Intramuscular	Ototoxicity, Nephrotoxicity	No

*Price based on preferential WHO-negotiated price.

1.4.3 Paromomycin

Paromomycin sulphate (PMM) is a parentally administered, aminoglycoside with a broad spectrum of action against bacteria, protozoans and cestodes. It was originally isolated from *Streptomyces* bacteria in 1950 and used as an antibiotic against both gram positive and negative bacteria (Davidson *et al.*, 2009). In eukaryotes, PMM is known to interact with subunits of ribosomal RNA and thus inhibit protein synthesis (Shalev *et al.*, 2013). In prokaryotes, PMM has been shown to interact with ribosomal RNA subunits and inhibit protein synthesis (Davis, 1988). The exact mechanism(s) and target of PMM in *Leishmania* are not fully understood and it is likely to be a multi factorial mode of action. Specifically in *L. donovani*, PMM has been demonstrated to target ribosomes, inhibit protein synthesis and disrupt mitochondrial membrane potential over a 72 hour period. Uptake of PMM

in to the cells was demonstrated to be non-carrier mediated and PMM likely binds to the glycocalyx of *Leishmania* (Jhingran *et al.*, 2009). It was further hypothesised that PMM enters in to the cell via endocytosis before accumulating in vesicles (Chawla *et al.*, 2011). Killing of *Leishmania* amastigotes was reported in murine models against CL as early as 1961 (Kellina, 1961). It wasn't until 1995 that activity against *L. donovani* was described *in vitro* (Neal *et al.*, 1995). Despite this PMM was already being used to treat travellers with VL returning to the UK from India and additionally, to cope with two epidemics in East Africa (Chunge *et al.*, 1990, Scott *et al.*, 1992, Thakur *et al.*, 1992). A retrospective study by Médecins sans Frontières (MSF), who had treated more than 67,000 patients over epidemic periods in Sudan between 1989 and 2000, assessed patient data and reported an initial cure rate of 97% (Melaku *et al.*, 2007). Phase II clinical trials in India evaluated both a 16 and 20 mg/kg/day regime for 21 days achieving 93% and 97% cure rates respectively. This showed significantly higher potency compared to the 63% cure rate from treatment with SSG at 20 mg/kg/day for 30 days, recommending that PMM replace SSG as the first line drug to treat VL in India, although this recommendation is yet to be implemented (Jha *et al.*, 1998). In 2007 PMM was registered as treatment for VL by the Indian government. A phase III trial in the same year compared the efficacy of PMM with that of AmB, the current first line treatment for VL in Bihar, India (Sundar *et al.*, 2007). Results indicated PMM to be non-inferior compared with AmB. This validated PMM as a viable alternative to the more expensive L-AmB in areas of SSG resistance. At approximately $10 per treatment (Table 1.1), PMM is the cheapest anti-leishmanial available and that, combined with its comparatively high efficacy

makes it a very attractive treatment option. Caution must be taken however as it has been shown that the standard regimen of paromomycin 15 mg/kg/day for 21 days was poorly efficacious in parts of East Africa, particularly Sudan (Hailu *et al.* 2010). Patients in Kenya and two locations in Ethiopia showed 80%, 75% and 96% final cure rates respectively, after 6 months. In contrast, patients in two locations of Sudan (Um El Kher and Kassaba) showed a significant reduction in efficacy, achieving only 14% and 46% cure rates (Hailu *et al.* 2010). A subsequent dose finding clinical trial assessing the effect of increasing the dose of PMM was then carried out (Musa *et al.* 2010). Treatment success was improved by increasing the duration of 15 mg/kg from 21 to 28 days and also by increasing the dose to 20 mg/kg for 21 days. Final cure rates of 85% and 90% respectively were achieved, therefore displaying a significant improvement. Additionally, a combination of PMM-SSG (15 mg/kg/day PMM and 20 mg/kg/day SSG) for 17 days has been shown to be comparable with a 30 day regimen of SSG (20 mg/kg/day) in East Africa (Musa *et al.* 2012). Similar to L-AmB, these results indicate geographical differences in the response to PMM treatment exist and imply that localised populations of parasites may respond differently to treatment. To avoid treatment failure and prevent acquired resistance emerging, dose guidelines should therefore be considered on the basis of the geographical location of the infection. Furthermore, it reinforces the need for close monitoring of PMM treatment, particularly in East Africa.

Absorption of PMM via oral administration is poor and thus treatment against VL must be administered intramuscularly. Administration at outpatient centres by trained healthcare workers is therefore essential, although unlike AmB, a prolonged

hospital stay is not necessary. The indirect costs of treatment are therefore relatively low. A 21 day regime of paromomycin costs approximately $10, making it the cheapest therapeutic option for the treatment of VL available (Davidson *et al.*, 2009). The low cost of the compound is mainly due to its orphan drug status granted by WHO in conjunction with the FDA and EMEA in the US and Europe respectively. With the low cost, comparable efficacy with L-AmB and without the need to remain in hospital, PMM is an attractive option and arguments could be made that it could replace both SSG and L-AmB as the first line drugs for treatment of VL.

1.4.4 Miltefosine

Miltefosine (hexadecylphosphocholine), marketed under the name Impavido® was the first, and remains to this day the only, oral drug available against VL (Ganguly, 2002). Originally synthesised in the late 1980s as an anti-tumour compound for the treatment of cancer, it also displayed efficacy against amastigotes of *L. donovani* (Croft *et al.*, 1987). MIL was eventually discarded from the anti-cancer trials due to poor bioavailability and cumulative toxicity *in vivo*. The WHO in partnership with ASTA Medica (later becoming Æterna Zentaris) subsequently initiated development and trials of MIL against VL. Initial clinical trials showed MIL to be active against VL which led to approval for the drug to be used to treat VL in India (Sundar and Murray, 2005). It also paved the way for a Phase II clinical trial in India (Sundar et al., 2002a). An oral MIL dose of 2.5 mg/kg for 28 days cured 94% of the 299 enrolled in the trial and moreover showed MIL to be non-inferior to the standard treatment

in India, AmB. The subsequent phase IV trial treating 971 individuals on the same course of MIL reported similar cure rates (95%) as the previous Phase III trial, supporting the use of MIL in India (Bhattacharya *et al.*, 2007). This dose of 2.5 mg/kg for 28 days remains the WHO guideline treatment against VL.

Although MIL is a very effective drug against VL there are a number of disadvantages associated with its use. The oral administration of the drug is somewhat of a double edged sword. It lessens the requirement of highly trained personnel or hospitalisation and thus helps reduce overall cost and treatment at out-patient clinics. This lack of supervision however, dictates that ensuring compliance with the full course of drug is difficult. Encouragingly, a recent study by Rijal *et al.* (2013) assessing the reason of relapse after MIL treatment recorded a compliance rate of >95% in a study, demonstrating that non-compliance with the full course of treatment may not be as low as expected (Rijal *et al.*, 2013). The fact that the patients were aware they were part of a trial however may have been a factor in a high compliance rate. MIL has also been associated with teratogenicity in animal models and therefore consideration must be taken when giving the drug to women of child bearing age. Contraception is therefore recommended to be used in conjunction with MIL treatment in such patients. This can have implications on the practicalities of both administration and additional costs, as contraceptives must also be taken in tandem with MIL.

Miltefosine appears to act on *Leishmania* in a number of ways including alterations in lipid metabolism, an inducer of apoptosis and by modulating the host

macrophage responses to kill the parasite. In humans, MIL has been characterised as an inhibitor of protein kinase B. Akt, as it is also known, is involved in a survival pathway in eukaryotes and inhibition leads to apoptosis in cells (Bononi *et al.*, 2011). A *Leishmania* analogue of Akt has not been identified so it would appear that MIL affects the parasite in an alternative yet unknown mechanism. Treatment of parasites has however been correlated with an increase in DNA fragmentation and nuclear condensation as well as a loss of membrane potential, indicating that apoptosis is still occurring (Moreira *et al.*, 2011). Reactive oxygen species have also been seen to increase with MIL treatment suggesting that oxidative stress is being induced in parallel. Several studies utilising liquid chromatography (LC) coupled with mass spectrometry (MS) have pointed towards lipid metabolism of *Leishmania* as a target of MIL. As early as 2002, it was postulated that MIL treatment impaired choline transport (Zufferey and Mamoun, 2002). Given that choline is a precursor to numerous lipid species found in the parasite, it is perhaps unsurprising that lipid metabolism would be affected by drug treatment. Levels of phosphatidylethanolamine (PE) and lysophosphatidylcholines (LPC) were also seen to increase in promastigotes treated with MIL (Rakotomanga *et al.*, 2007). A more detailed study by Imbert *et al.* identified several classes of lipids that were perturbed after treatment of promastigotes with MIL. These included; PEs, LPCs, phosphatidylcholines (PC), phosphatidylinositols (PI), phosphatidylserines (PS) (Imbert *et al.*, 2012). Although individual lipids differed, in general, MIL treatment caused a massive decrease in PC content of promastigotes in tandem with a less pronounced but nevertheless conspicuous increase in PE and PI content. A more

recent study (Vincent *et al.*, 2014) studied the effects of MIL on *L. infantum* and brought together several parts of the earlier work. Almost 4 hours post treatment; ROS were seen to increase in parallel with an increase in the levels of several sugars and alkanes. In fact, almost 10% of the total metabolome of *L. infantum* had been significantly altered. It was also reported that after five hours, parasite membranes were critically damaged and leaking a variety of metabolites. Alteration in phospholipid biosynthesis and flagellar membrane damage has also been reported in *T. brucei* and PC synthesis inhibition in tumour cells via inhibition of the phosphatidylcholine cytidylyltransferase enzyme has been characterised (Vincent *et al.*, 2014).

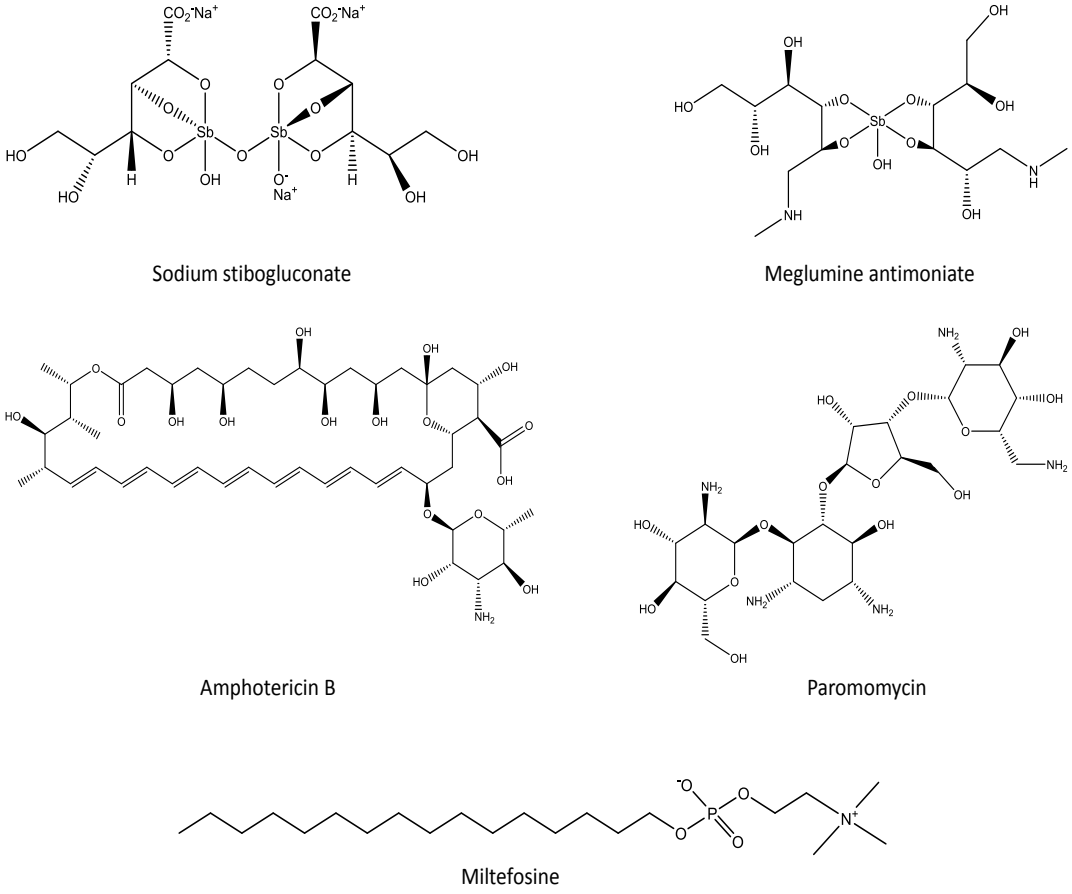


Figure 1.4. Chemical structures of drugs currently used to treat VL. Drawn using ChemBio Draw Ultra 14.0

1.5 Drug Resistance in VL

With the limited number of available treatments against VL, the emergence of drug resistant *L. donovani* is a major concern. At present, only resistance to Sb has been reported in the field however the possibility of acquired resistance in the laboratory has been demonstrated for all the alternative drugs.

The majority of *Leishmania spp.* have zoonotic lifecycles, capable of being transmitted between humans and other mammals, such as dogs, via sandflies. On the other hand, *L. donovani* is restricted to only human-sandfly-human transmission, an anthroponotic lifecycle (Magill, 1995). This is significant as it impacts upon the rate at which drug resistance can emerge and proliferate. An anthroponotic lifecycle means that the amastigote parasite is continually in a human host and more likely to be subjected to drug treatment. This increases the selective pressure of chemotherapy, positively selecting parasites less susceptible to the chemotherapy and allowing proliferation of resistant parasites. In contrast, while a zoonotic lifecycle puts less pressure on parasites as the can stay in a canine reservoir host, reducing the chances of coming in to contact with chemotherapy.

Co-infections of HIV and leishmaniasis are another factor that can contribute to the emergence of drug resistant *L. donovani*, particularly in East Africa where Human immunodeficiency Virus (HIV) rates are high. HIV patients infected with *L. donovani* typically have higher than normal parasite burdens and either respond slowly to treatment or don't respond at all to the recommended doses. Incidences of relapse

are also significantly higher in HIV positive patients (Laguna *et al.*, 2003, Russo *et al.*, 1996).

1.5.1 Sodium Stibogluconate

In use since the 1940s, the emergence of SSG resistance was first documented in the 1980s, when Peters reported unresponsiveness in 30% of patients treated with a dose of 10 mg Sb^V/kg SSG for 10 days (Peters, 1981). As a result, the recommended treatment was increased to a period of 20 days and eventually to a dose of 20 mg Sb^V/kg for 20 days. Gradually, unresponsiveness to this dose began to increase and thus the recommended regimen was increased again. This cycle of treatment failure and increased dosage has continued in Bihar. Between 1997 and 2000, the actual cure rate using a dose of 20 mg Sb^V/kg over a period of 30 days was as low as 30% of patients (Sundar *et al.*, 2000, Sundar *et al.*, 1997b). Interestingly, in one of those studies a stark contrast in treatment failure rates between neighbouring districts became apparent. While unresponsiveness in patients in the whole Bihar district average 60%, those in the district of Uttar Pradesh showed only 2% unresponsiveness (Sundar *et al.*, 2000). It has been suggested that treatment failure was due to acquired drug resistance over time, rather than as a consequence of different strains of parasites (Lira et al., 1999b) but genotyping studies would be required to confirm this assumption. A similar study by (Thakur *et al.*, 2004) reported that even in neighbouring districts of Bihar, unresponsiveness was unpredictable and could vary between 0% to 100% of treatment failure, further evidence that different populations of parasites had acquired resistance. To test

this, Lira *et al.* tested the susceptibility of parasites to SSG from patients who responded to the drug and patients who did not respond. Parasites taken from responsive patients were over 3 times more sensitive to SSG than those taken from non-responders (Lira et al., 1999a). This observation supported the hypothesis that the underlying cause of SSG resistance is due to an acquired resistance by the parasite.

One of the key elements in the acquired resistance of SSG was extensive misuse of the drug in Bihar although an alternative theory that postulates that arsenic-tainted drinking water may also have contributed to parasitic tolerance (Nagle et al., 2014). Several studies have also highlighted the lack of regulation in medical practitioners in the Bihar also having an impact of the development of resistance (Sarnoff et al., 2010, Sundar and Chakravarty, 2010). Guidelines for correct dosing and treatment duration were not followed correctly and often sub efficacious doses were prescribed. This is possible because in India prescriptions are often not required and patients can buy as little or as much of a drug as they like. This creates the scenario where patients will buy a drug until they begin to feel better, at which point they will stop treatment in an effort to save money. Such practices provide ideal conditions for acquired resistance to emerge and become widespread as parasites are subjected to sub-lethal doses of SSG. One study showed that >70% of VL patients had consulted unqualified doctors as their first port of call (Sundar *et al.*, 1994). Moreover, of those analysed, only 26% were treated in line with WHO guidelines and over one third of these patients stopped treatment of their own accord. At the same time, the production of SSG is poorly regulated, creating

inconsistencies between batches of SSG leading to documented incidences of severe toxicity in patients (Sundar *et al.*, 1998). In areas of East Africa however, a 30 day course of SSG remains effective (> 90% cure rate), showing that there is still a use for the drug in the fight against VL in certain geographical locations (Musa *et al.* 2012.

The mechanisms of SSG resistance has been shown to be multi factorial. Shaked-Mishan *et al.* have shown in laboratory models that parasites resistant to SSG have a significantly deficiency in their capacity to reduce Sb^{V} to the active Sb^{III} molecule (Shaked-Mishan *et al.*, 2001). An increase in production of γ-glutamylcysteine and the TSH precursor ornithine decarboxylase as well as increased TSH levels has also been shown to correlate with an increase in tolerance to SSG (Grondin *et al.*, 1997, Mukhopadhyay *et al.*, 1996). Such an increase in TSH levels would help to offset that lost from the cell and restore the thiol redox potential. Aquaglycoporins, known to uptake Sb^{III} into the parasite cell have also been implicated in having a possible role in parasite resistance to SSG (Maharjan *et al.*, 2008). Conclusive evidence for either mechanism is as yet elusive and the exact mechanisms of parasite resistance to SSG treatment are unknown.

1.5.2 Amphotericin B

With incidences of SSG resistance increasing so too is the reliance on other drugs such as AmB as an alternative treatment. For this reason, it is imperative that use of AmB is closely monitored and controlled to avoid misuse and occurrences of resistant parasites. Encouragingly, incidences of AmB resistance in fungi are low

which would imply in some way that it may also be difficult for *Leishmania* to become resistant. A report focusing on 5 year field reports of the long term effectiveness of L-AmB in Bihar found a small number (2%) of relapse cases after 15 months post treatment with AmBisome (Burza *et al.* 2014). Despite this relatively low relapse rate, resistance can be selected for relatively easily in the laboratory (Mbongo et al., 1998b). In these isolates, a change in the parasites plasma membrane composition from ergosterol to the cholesterol, cholesta-5,7,24-trien-3β-ol, has been described. The mechanism for this alteration has been proposed as being due to a loss of function of S-adenosyl-L-methioninesterol transferase enzyme. Transcripts for the enzyme have been shown to be either missing or not translated in *L. donovani* lines resistant to AmB as well as *Candida spp.* (Pourshafie *et al.*, 2004, Ishida *et al.*, 2009). The geographical differences in the required doses for cure have been reported which suggest there could be strain dependent susceptibilities in the response to AmB. Patients in India require much lower doses of AmB compared to those in Africa (Sundar *et al.*, 1997a, Sundar *et al.*, 2002c).

1.5.3 Paromomycin

Widespread field resistance of *L. donovani* to PMM has not yet been found but a small number of clinical isolates with variable susceptibilities to PMM have been reported as far back as 1995 (Neal *et al* 1995). Interestingly, in one example paromomycin resistant (PMM-R) parasites were isolated from a patient who had no known history of PMM treatment, suggesting that naturally resistant parasites exist (Hendrickx *et al.*, 2014). To date, all studies examining the mechanisms of PMM

resistance have been performed on laboratory generated resistant lines. PMM resistance appears to be underpinned by a decreased accumulation of the drug inside the cell, coupled with an increased protection against macrophage killing mechanisms that are induced by the drug. The decreased accumulation of PMM inside the cell of resistant parasites appears have multiple convergent mechanisms. The first evidence correlating PMM resistance with a decrease of internalised PMM was in 1998 (Maarouf *et al.*, 1998) but it wasn't until 2014 when Bhandari *et al.* evaluated the expression of drug transporters, that a cause was identified (Bhandari *et al.*, 2014). Expression of the ABC transporters MDR1 and MRPA and regulatory protein, protein phosphatase 2A were all seen to be greatly increased in PMM resistant promastigotes. MDR1 and MRPA are known to be important for xenobiotic detoxification and efflux of metabolites from the cell (Bhandari *et al.*, 2014). It was hypothesised that PMM resistance is at least partially mediated via an efflux of the drug from the cell. PMM resistant parasites have also been characterised as having an increased tolerance to ROS which is known to be induced by PMM (Berg *et al.*, 2015). A recent metabolomic analysis of PMM resistant promastigotes elucidated that an increase in proline biosynthesis, in parallel with an increase in tryptophan degradation as a possible mechanism behind this increased resistance to cell killing (Berg *et al.*, 2015). Proline has been shown to protect cells, including *T. brucei* and *Leishmania spp.* against the effects of ROS. Berg *et al.* measured significant increases in in proline and metabolites involved in its biosynthetic pathway as well as metabolites involved in the transsulfuration pathway.

1.5.4 Miltefosine

To date, there have been no reported incidences of resistance to MIL although several cases of relapse in MIL treated patients have been described. These, along with a number of other factors make resistance possible and it is thought that it is only a matter of time until resistance appears (Dorlo *et al.*, 2012). Failure to complete regimens of chemotherapy can be a factor in the emergence of resistance and must be taken seriously, particularly with MIL. Non-compliance of treatment guidelines means that parasites are exposed to sub-lethal doses of drug and can become desensitised to it effects. MIL has a very long terminal half-life, lasting between 150-200 hours, resulting in it taking up to 33 days to reach a clearance of >95% in the body (Bryceson, 2001). As a consequence, levels of MIL are present in the body long after treatment with the drug has ceased. In a 171 patient study only 83% of patients adhered to the correct treatment protocol (Uranw *et al.*, 2013). The oral administration of MIL also means that compliance with the full course of the drug is harder to guarantee as supervision of administration is not necessary. Worryingly, the efficacy of MIL in the ISC appears to be waning in both India (Sundar *et al.*, 2012) and Nepal (Rijal *et al.*, 2013) with repots of relapses after MIL treatment occurring. Interestingly, characterising the sensitivity to MIL of parasites isolated from these relapse patients did not show a decreased susceptibility to the drug. With the lack of other options currently available or in the pipeline for VL treatment, extending the clinical life of MIL is of major importance and resistance to MIL has been widely studies in the laboratory. Thus far, laboratory selected MIL resistance has always associated with decreased drug accumulation within the

parasite, via one of two mechanisms. The first involves a plasma membrane P-type transporter from the aminophospholipid translocase subfamily. This transporter has been characterised to uptake MIL as well as other glycerophospholipids and has been termed the *L. donovani* miltefosine transporter (LdMT). Two point mutations identified on separate alleles were first identified as being responsible for a reduced uptake of MIL in *L. donovani* (Perez-Victoria *et al.*, 2003b). Later, it was also shown that alteration to a beta subunit of LdMT belonging to the CDC50/Lem3 protein family, termed LdRoS could also play a role in resistance to MIL (Perez-Victoria *et al.*, 2006). It is therefore apparent that both proteins are mutually dependent on each other for proper localisation and functioning of the LdMT. Deletions or mutations in the genes encoding either of these proteins leads to a translocation of the other protein and have been implicated with reduced MIL uptake and subsequent MIL resistance (Perez-Victoria *et al.*, 2006, Perez-Victoria *et al.*, 2003b).The importance of the LdMT gene in drug resistant *L. major* has also demonstrated (Coelho *et al.*, 2012a). The second mechanism that can lead to a reduction of internalised MIL in the parasites is via an increased efflux of the drug. Over expression of P-glycoprotein of the ABC family of transporters in *L. tropica* has previously been associated with a resistance to MIL although as yet, this has not been reported in *L. donovani* (Perez-Victoria *et al.*, 2001, Seifert *et al.*, 2003).

1.5.5 Socio Economics of VL Treatment

Several factors such as the anthroponotic transmission of *L. donovani* VL or mechanisms of particular drugs can increase the likelihood of drug resistance

developing. Another important consideration is the socio-economics of the people who live in VL endemic areas. Ninety percent of all VL cases occur in India and Nepal and the majority of these cases are confined to the Bihar region of India. A 2010 study on the economic impact of VL in Bihar detailed the severe economic impact that VL can have upon people living in this region (Sarnoff *et al.*, 2010). With an average daily wage of $1, the impact of VL represents a loss of over 7 months of household earnings. For comparison, the average wage of a UK worker is £74 per day before tax (http://www.ons.gov.uk last accessed 01/02/2016). Moreover, a UK resident has access to the National Health Service that provides free healthcare. Sarnoff's analysis also reported that 87% of VL affected households had to take out loans in order to finance treatment of the disease. If treating a disease occupies 7 months of household wages and necessitates a loan being taken out to pay for treatment then it is understandable that efforts to recoup expenditure will be made. In cases like this, antileishmanial drugs are often shared between multiple people or sold within the community so the full course of treatment will not be followed (Prof. Mike Barrett personal communication). Another important statistic highlighted in the Sarnoff study is that 40% of patients sought the aid of unqualified "non-physician rural practitioners" with the end result of less than 3% of patients being correctly diagnosed with VL. A similar study reported that patients seeking unqualified medical practitioners as their initial examination was as high as 80% of patients (Hasker *et al.*, 2010). Misdiagnosis or treatment using either ineffective chemotherapy (i.e. Sb use in Bihar) or incorrect dosing of drugs has the potential to significantly increase the incidence of resistance. Parasites exposed to sub lethal

doses of drugs via incorrect dosing or cutting their treatment short creates conditions that aid parasitic resistance developing. Anti-leishmanials can also be bought over the counter in India and counterfeit drugs with little or no active ingredient are found which can compromise compliance and correct dosing of chemotherapy (Kaur *et al.* 2014) (Dorlo *et al.*, 2012, den Boer *et al.*, 2011).

With these facts in mind, resistance to VL chemotherapy is likely to continue to be a problem. VL treatment and resistance is a complex and fluid problem that cannot be addressed easily. It is not just a case of finding an effective, easily administered drug that will eradicate VL. Even reducing the cost of treatment will still leave it unaffordable to swathes of people, purely because of other treatment costs. Resistance has made SSG obsolete in the ISC and it is likely to impact the other existing chemotherapy, particularly MIL. Better understanding the currently available chemotherapy and how resistance mechanisms may manifest and impact on overall parasite fitness is therefore crucial to preventing its occurrence and prolonging current chemotherapy.

1.6 Phospholipid Metabolism in *Leishmania*

The plasma membrane of *Leishmania* is either a direct target of or significantly affected by treatment or resistance to AmB, PMM and MIL. In order to understand the implications of the mode of action of drugs and resistance mechanisms, an understanding of the plasma membrane and its composition is vital. The plasma membrane is the structure responsible for separating the interior of the cell with the exterior environment and controls the movement of substance in and out of the

cell. It is composed mainly of a phospholipid bilayer interspersed with proteins and glycoproteins. The phospholipids arrange themselves in a bilayer consisting of hydrophilic head region oriented outwards in contact with the cell cytosol and exterior environment, while the hydrophobic tails point inwards towards the interior of the bilayer. Phospholipids are therefore integral to the plasma membrane and any change in their content or metabolism can have significant consequences in cells.

1.6.1 Phospholipids

Lipids compose approximately 25% of the total *Leishmania* metabolome and phospholipids (PLs) account for 70% of the total lipid content [reviewed by (Zhang and Beverley, 2010, Smith and Butikofer, 2010)]. Phospholipids are a class of lipids that are a major component of membrane composition and play various roles in cell signalling. Importantly, alterations in membrane fluidity and PL composition of the parasite membrane have been associated with resistance to both MIL and PMM (Li *et al.*, 2006). The majority of phospholipids are comprised of a phosphate group, a glycerol unit, two fatty acid chains and a simple polar molecule such as choline, ethanolamine or serine. The only phospholipid exception to this structure is sphingomyelin, where the glycerol is replaced by a sphingosine (Figure 1.5). PLs can be classified based on the polar headgroup that they contain with the main groups being; phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylinositols (PIs) and sphingolipids (SLs) (Zhang and Beverley, 2010).The bonds that attach the fatty acid tails to the glycerol backbone can also be used to

further classify members of each group. The three varieties are; two acyl links (1, 2-diacyl), and acyl and an alkyl (1-alkyl-2-acyl), or an acyl and an alkenyl (1-alkenyl-2-acyl). PLs with one of their fatty acid tails cleaved are referred to as lyso-PLs.

Structurally, the polar molecule and phosphate unit will form a hydrophilic head region while the fatty acid tail is hydrophobic. In aqueous environments, phospholipids form a bi-layer with the hydrophilic heads point orienting themselves on the outside of the bi-layer while the fatty acid tails point inwards towards each other. The composition of PLs present can dramatically alter the characteristics, particularly the fluidity of a plasma membrane. In general, the higher the PC: PE content the less fluid the membrane (Li *et al.*, 2006). Likewise, longer chain PLs and a higher number of saturated carbon to carbon bonds in fatty acid tails decrease the fluidity of the plasma membrane (Rakotomanga *et al.*, 2007).

Phosphatidylcholines are the most abundant PL within *Leishmania*. Ninety five percent of choline in cells is incorporated within PCs (Dowd *et al.*, 2001). PCs are predominantly found with long, unsaturated fatty acid tails. As with most PLs, they have been shown to have a range of functions in membrane structure and signalling. Changes within the PC composition are associated with altering membrane melting point and fluidity while also having a role in resistance to oxidants. The phosphocholine component of PCs is an important mitogen, essential in the synthesis of DNA (Li and Vance, 2008). Likewise, phosphatidylethanolamines (PE) are important in cell signalling and membrane structure where they are known to regulate membrane curvature. In *L. major*, the major PE found is

plasmenylethanolamine (1-alkenyl-2-acyl-phosphotidylethanolamine, which accounts for approximately 85% of the total PE content in the parasite (Zhang and Beverley, 2010).

1.6.2 Synthesis of PC & PEs

Lipid metabolism within *L. donovani* is not fully understood as yet. Some aspects are inferred from work done on similar pathways in other *Leishmania spp.* or other kinetoplastids such as trypanosomes. Significant differences between *Leishmania* and trypanosomes however are apparent and so any data inferred must be used with caution. For example, *Leishmania* have been shown to uptake choline (Zufferey and Mamoun, 2002) whereas trypanosomes acquire it through the uptake and catabolism of lyso-PCs (Rifkin *et al.*, 1995). Both kinetoplastids have been demonstrated to uptake ethanolamine and inositol however (Vince *et al.*, 2011, Seyfang and Landfear, 2000, Bibis *et al.*, 2014). Likewise, different *Leishmania spp.* cause dramatically different diseases and unsurprisingly, metabolomic differences between species was recently detailed (Westrop et al., 2015).

The Kennedy Pathway (Figure. 1.6) is an aminoalcoholphosphotransferase reaction responsible for the de novo synthesis of PC and PE within *Leishmania* (Zhang and Beverley, 2010, Smith and Butikofer, 2010). It is named after Eugene Kennedy who first described it in 1956. Two separate branches of the pathway synthesis either PC or PE from either choline or ethanolamine respectively.

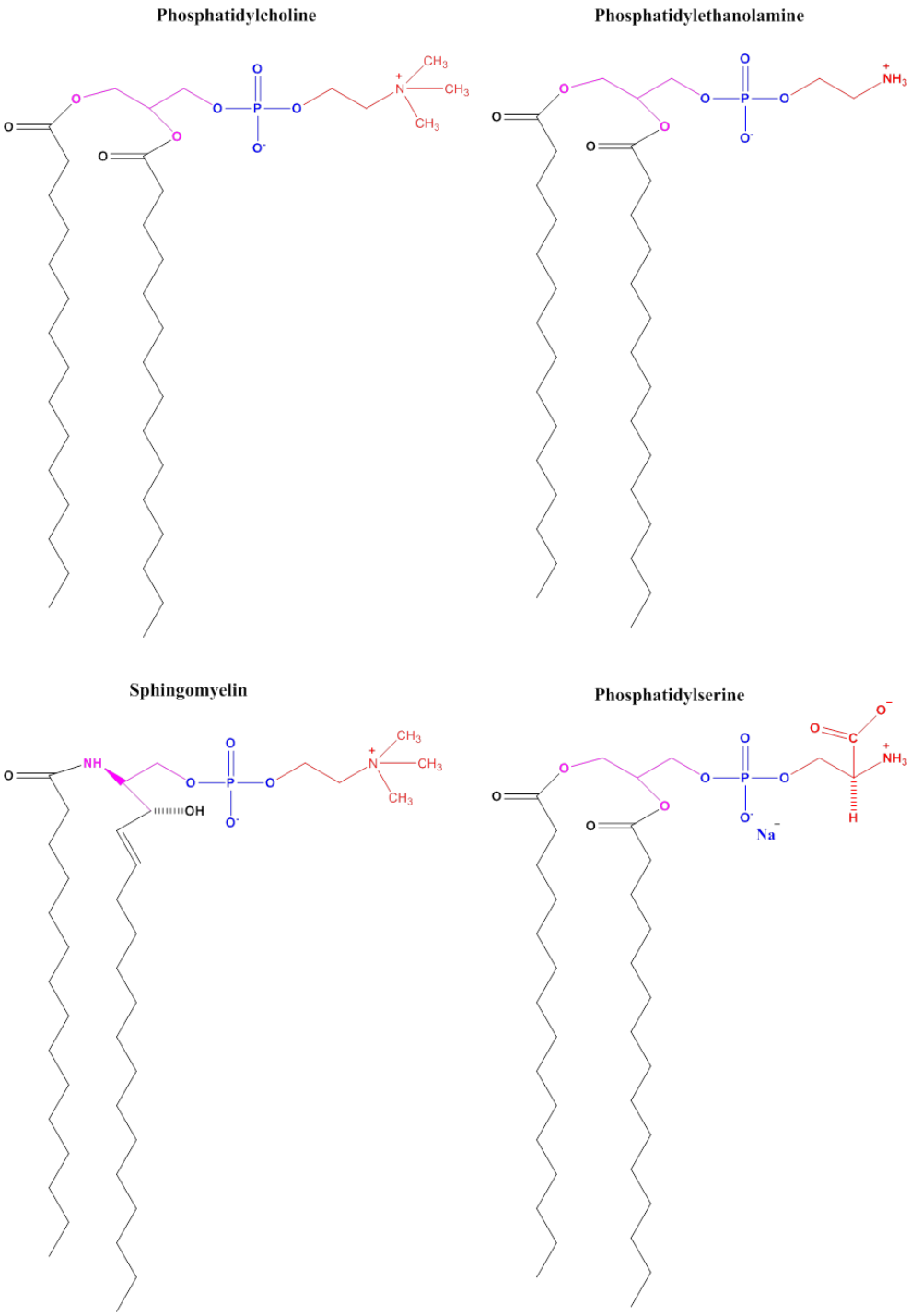


Figure 1.5. Structure of common phospholipids found in *L. donovani*. Colour code is as follows; Fatty acid tails (black), glycerol (pink), phosphate (blue) and polar head group (red).

The first step in the choline pathway is the phosphorylation of choline by a choline kinase to produce choline phosphate. Cytidine diphosphate (CDP)-choline is produced via the enzymatic addition of a cytidine-phosphate molecule, mediated via choline transferase. A di-acyl-glycerol headgroup is then transferred to CDP-choline via choline phosphotransferase to produce the final phosphatidylcholine. Synthesis of PE is almost identical with the exception of ethanolamine replacing choline as the initial substrate. The majority of enzymes of the pathway are able to overlap and catalyse steps on either branch of the Kennedy Pathway. Choline kinase, for example, has been shown capable of catalysing the reaction of choline to choline-phosphate and ethanolamine to ethanolamine-phosphate. The two cytidylyltransferase enzymes (CCT and ECT) are however highly specific. It is this step, the addition of the cytidine-diphosphate moiety that is the rate limiting step of the Kennedy Pathway (Smith and Butikofer, 2010). Phosphatidylcholines and phosphatidylethanolamines are therefore very important components, not only of the *Leishmania* plasma membrane but as metabolites of the parasite in general. With drugs such as AmB, PMM or MIL that are known to associate with the parasite's plasma membrane or specific parasite lipids, expanding our understanding of PC and PE synthesis and how these lipids are altered upon acquiescence of drug resistance would be hugely beneficial.

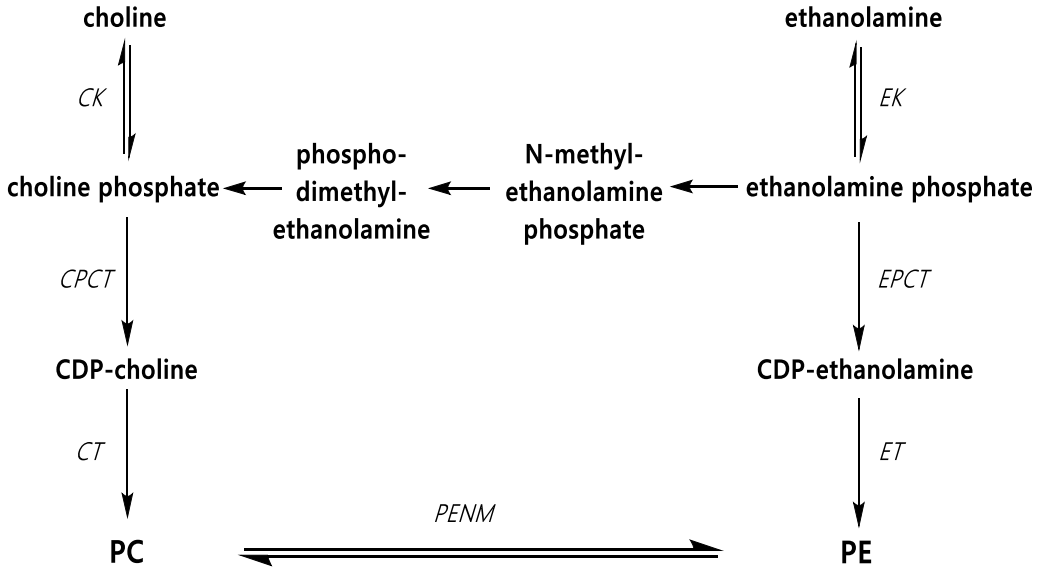


Figure 1.6. The predicted Kennedy pathway in *Leishmania spp.* Enzymes are abbreviated, in italics and include CK (choline kinase), EK (ethanolamine kinase), CPCT (choline-phosphate cytidylyltransferase), EPCT (ethanolamine-phosphate cytidylyltransferase), CT (choline phosphotransferase), ET (ethanolamine phosphotransferase), PENM (phosphatidylethanolamine N-methyltransferase).

1.7 Aims of this study

There are currently only a limited number of chemotherapy choices available to treat VL. Antimonials have been used as a treatment for over 80 years but due to acquired resistance, their use is now limited in the ISC. Despite use of antimonials being abandoned over 20 years ago, Sb resistant populations are still found in endemic regions (Vanaerschot et al., 2014). Other options such as MIL or PMM are currently effective against *L. donovani* but the threat of resistance emerging is a distinct possibility (Rijal et al., 2013). What impact, if any, the antimony susceptibility of an isolate has upon the likelihood and outcome of resistance to other drugs developing is not known. Mapping changes that occur in the metabolome of parasites that have acquired resistance to MIL or PMM and linking these changes to genomic profiling would help assess the mechanisms behind this acquired resistance. Additionally, assessing the impact of this acquired resistance to the overall fitness of cloned, drug resistant parasites will allow an insight in to the potential of these drug resistant parasites to outcompete drug susceptible populations. Therefore, the aim of this study is to:

- Characterise and link differences in the genome and metabolome of *L. donovani* clinical isolates with different inherent susceptibilities to antimony
- Select for resistance to miltefosine or paromomycin in these clinical isolates via a stepwise increase in drug pressure and clone resulting drug resistant parasites

- Analyse the metabolome of miltefosine resistant promastigotes to identify changes associated with acquired resistance and correlate with observed genetic alterations
- Assess the impact of selected drug resistance on the fitness of drug resistant parasites by measuring; infectivity and proliferation both as promastigotes and amastigotes as well as the ability of intracellular amastigotes to modulate macrophage cytokines.
- Analyse the metabolome of paromomycin resistant promastigotes to identify changes associated with acquired resistance and correlate with observed genetic alterations
- Screen a small library of novel minor groove binding molecules, synthesised at the University of Strathclyde, for efficacy against *L. donovani in vitro* and test and suitable candidates for activity against the parasite *in vivo* using Balb/c mice

Chapter 2. Materials & Methods

2.1 Reagents

Sodium stibogluconate (Lot no. 266402A) and miltefosine (Batch 1149149) were provided by WHO-TDR. Paromomycin was obtained from Sigma-Aldrich. HOMEM medium, molecular water and Trypan Blue were supplied by Gibco, Paisley, UK. RPMI, 1640 medium and Foetal Bovine Serum Gold were acquired from PAA Laboratories, Yeovil, UK. Ammonium carbonate, ammonium formate, resazurin salt, Giemsa solution, formaldehyde and DPX mountant were purchased from Sigma Alridrich, Gillingham, UK. Dulbecco's Modified Eagle Medium (Lonza, Huddersfield, UK). D-Luciferin potassium salt was supplied by Caliper Life Science, Runcorn, UK. Chloroform was obtained from Alfa Aesar, Heysham, UK. HPLC grade methanol, water, acetonitrile and isopropyl alcohol was purchased from Fisher Scientific, Loughborough UK. DNeasy Blood & Tissue kit and RNeasy Mini Kit were obtained from Qiagen, Crawley, UK. MyTaq DNA Polymerase and random primers were supplied by Bioline Reagents Ltd, London, UK. AffinityScript Multiple Temperature Reverse Transcriptase was purchased from Stratagene, UK. SYBRgreen Absolute PCR was obtained from Abgene, Thermo Scientific, Hemel Heampstead, UK. Mass spectrometry vials and accompanying low-volume inserts were purchased from Kinesis, St. Neots, UK. The SeQuant ZIC®-pHILIC column was supplied by Merck Millipore, Watford, UK and the Silica gel column was obtained from HiChrom, Reading, UK. All other reagents were of analytical grade.

2.2 Mice

Sex matched BALB/c female mice, at six to eight weeks old, bred in house at University of Strathclyde were used in this study. Animal experiments were carried

out in accordance with local ethical rules, within Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines and under United Kingdom Home Office regulations.

2.3 Parasites

L. donovani strains with different Sb susceptibility backgrounds were derived from patients at the B.P. Koirala Institute of Health Sciences, Dharan, Nepal; MHOM/NP/02/BPK282/0cl4 (Sb sensitive, Sb-S), MHOM/NP/02/BPK087/0cl11 (Sb intermediate resistant, Sb-I) and MHOM/NP/02/BPK275/0cl18 (Sb resistant, Sb-R). Where possible, passage numbers of WT and their drug resistant parasites were matched in order to minimise the effects of continuous culturing on experimental data generated. After selection of drug resistance and cloning, the passage number of drug resistant parasites were as follows; Sb-S MIL-R 64 (48), Sb-R MIL-R 72 (40), Sb-S PMM-R 68 (44), SbI PMM-R 58 (46) and Sb-R PMM-R 69 (37), where the first number represents the number of passages since isolation from the patient and the number in brackets corresponding to passages since the isolate was originally cloned. Promastigotes were continually cultured in HOMEM supplemented with 20% v/v foetal calf/bovine with the exception of using Locke Medium on Tobie's agar base when cloning parasites. For bioluminescence studies, two clones of *L. donovani* strain MHON/ET/67:LV82, designated Luc 1.2 and 2.2, made by Dr. Rodderick Williams at University of Strathclyde, were used. Luciferase parasites contained the integrative construct, designated pGL1313 (9475 bp), was a gift from Prof. D. F. Smith which contained pSSU-int fragments to facilitate integration into the *Leishmania* genome via the ribosomal RNA small subunit, and is therefore

constantly expressed. The vector also contained a hygromycin resistance marker. Data on expression levels and correlation between number of parasites and bioluminescence emitted are discussed later in section 8.2.1.

Promastigotes were maintained in complete HOMEM supplemented with 20% v/v foetal calf/bovine serum at 26°C. Cultures were seeded using 2 x 10^5 parasites/ml in 5ml of culture medium and sub-passaged every week into a new flask. Miltefosine and paromomycin resistant parasites were cultured with 74 µM and 96 µM of miltefosine or paromomycin respectively. To determine parasite numbers, cultures were diluted in an equal volume of 99% PBS pH 7.4: 1% formaldehyde and 10µl was loaded into a Neubauer haemocytometer and the number of parasites/ml was determined by viewing under a microscope at x400 magnification.

2.4 Induction of drug resistant parasites

Induction of resistance to MIL or PMM was carried out on the promastigote stage of *L. donovani* parasites. Strains Sb-S and Sb-R parasites were grown in step-wise increasing concentrations of MIL (3, 6, 12.2, 35, 49.2, 61 and 74µM) in HOMEM 20% v/v foetal calf serum (FCS) until parasites grew at a similar rate as WT parasites. Parasites of the Sb-S and Sb-R strains able to grow in 74 µM MIL were designated as MIL resistant (MIL-R) parasites. The maximum concentration of MIL used to culture promastigotes was 74µM. Above this concentration, levels of MIL were observed to be toxic to host macrophages used in amastigote studies. Adapting WT strains to the highest concentration of MIL took a total of 31 weeks of incremental drug pressure. All work to select MIL resistance was carried out by Dr Julien Lonchamp at

Strathclyde University. Resistance to PMM was selected for by Craig Shaw as described for MIL above. Strains Sb-S, Sb-I and Sb-R were exposed to step-wise increments of 2, 4, 8, 16, 32, 64 and 97µM PMM over 26 weeks to create paromomycin resistant (PMM-R) strains. Above 97µM, parasite growth did not replicate WT growth.

2.5 Cloning of parasites

Cloning of *L. donovani* strains Sb-S and Sb-R MIL-R parasites was attempted using two methods; a dilution method and a hanging drop method under aseptic conditions.

For the dilution method of cloning, day 4 cultures were made to a concentration of $1x10^6$ parasites per ml in complete HOMEM 20% FCS. A 1 ml sample was diluted 1:25 to give $4x10^4$ cells/ml. This process was repeated twice more to produce a dilution of 64 parasites/ml from this dilution, 200 µl was added in to each well of a 96 well plate and incubated at 26°C for at least 2 weeks.

Prior to cloning using the hanging drop method, parasites were adapted to grow in Locke Medium (137mM NaCl, 3mM KCl, 2mM KH_2PO_4, 0.5mM $MgSO_4.7H_2O$, 2mM $NaHCO_3$, 14mM Glucose, 200,000 IU/L Penicillin, 200 mg/L Streptomycin, pH 7.4) on top of Tobie's Blood agar base (15% w/v Bacto-Agar, 15% w/v Bacto-Tryptose, 68mM NaCl, 13mM $Na_3PO_4.12H_2O$, 5mM KCl) for at least one week. At cloning, a drop of parasite culture was added to 1.5 ml Locke medium. A micro drop of this suspension was spotted onto a 22 x 22 mm glass slide using a micro-pipette, and the coverslip was then placed face down into the cavity of a glass microscope cavity

slide and sealed with complete HOMEM. The micro drop was checked for the presence of a single parasite under x200 and x400 magnification. The sample was discarded if more than one parasite was present and dilution of parasite suspension adjusted accordingly. The presence of a single parasite was confirmed by a 2nd observer. Drops containing a single parasite were washed into the cavity of the slide with 200µl conditioned Locke using a 25G needle and 1 ml syringe and transferred into a 14 ml Cellstar® (Greiner Bio One, Belgium) culture tube containing 2 ml of Tobies's blood agar. The syringe was washed with a further 300 µl of conditioned Locke and the contents added to the same culture tube as before. Ten clones were set up per session for each *L donovani* MIL-R/PMM-R strain. Cloned parasites were incubated at 26°C, with the culture tube at a 45° angle, for a maximum of four weeks and checked weekly to monitor parasite growth. A further 0.5 ml Locke was added when medium needed replenishing and cultures were left to grow for a further week before passaging as normal.

2.6 Macrophages

Peritoneal and bone marrow macrophages were obtained aseptically from BALB/c female mice at six to eight weeks of age. Mice were sacrificed and the surface of their front washed with 70% v/v aqueous ethanol. Peritoneal macrophages were harvested by injecting the peritoneal cavity of an individual mouse with 3 ml incomplete RPMI1640 (supplemented with 100 mg/ml penicillin/streptomycin, 100 mg/ml L-glutamine). The abdomen was gently massaged to detach macrophages and extracted using a 25 gauge needle. Bone marrow macrophages were obtained by flushing out bone marrow from individual mouse femurs with 5 ml of bone

marrow medium (Dulbecco's Modified Eagle Medium supplemented with 30% v/v L-cell supernatant, 20% v/v FCS**,** 100mg/ml penicillin/streptomycin and 100 mg/ml L-glutamine), using a 25G needle attached to a 10 ml syringe. The resulting bone marrow suspension was incubated for one week in Petri dishes at 37°C in an atmosphere of 95% air; 5% CO_2 to allow differentiation into bone marrow macrophages. Before adding to tissue culture plates, macrophages were incubated at 4°C for 5 minutes in fresh Dulbecco's Modified Eagle Medium and scraped using a cell scraper to remove adhered cells from the petri dish. Macrophages were then pooled and centrifuged at 2700 x g for 5 minutes. The resulting cell pellet was washed twice in incomplete RPMI 1640, resuspended in complete RPMI 1640 (supplemented with 10% v/v FCS). The number of viable macrophages was determined by diluting a sample of the macrophage suspension 1:1 with Trypan Blue solution which selectively stains dead cells blue A 10 µl sample was loaded into a Neubauer haemocytometer and the number of viable cells/ml determined microscopically at x400 magnification. In all cases the percentage of viable cells was always >95%.

2.7 Determining infectivity of parasites

Macrophages were infected with the relevant *L donovani* parasite at different parasite:host cell ratios to determine parasite infectivity. On Day 0, macrophages ($0.5x10^5$ cells/well, 0.2 ml) in complete RPMI were added to the appropriate wells of a 24-well plate containing a 13 mm^2 circular glass coverslip. Plates were incubated overnight at 37°C in an atmosphere of 5% CO_2; 95% air to allow macrophages to adhere. *L. donovani* promastigote parasites of the appropriate

strain/clone were harvested from a culture, resuspended in complete RPMI 1640 medium, and the number of promastigotes/ml determined. Parasites were added to the appropriate wells of each plate on day 1 at a host cell: parasite ratio of 1:5, 1: 10, 1:20 or 1:40 (n = 4/treatment). Plates were then incubated as before to allow parasites to invade macrophages. On day 2, the medium from each well was replaced with 0.5 ml complete RPMI1640 medium per well to remove unattached parasites. The plate was incubated as before for 72 hours. The medium was then removed from each well and approximately 200 µL of 100% methanol was added to each well. Plates were incubated for 5 minutes before the methanol removed and replaced with 10% v/v aqueous Giemsa solution for 20 minutes to stain. Coverslips were washed twice using water, removed from plates and left face up to air dry overnight. Coverslips were then mounted onto glass microscope slides using DPX mountant. The coverslips were viewed under x100 magnification and the percentage of infected macrophages/coverslip determined from 200 random macrophages. The number of amastigotes inside 20 randomly chosen infected macrophages was also determined to calculate number of parasites/infected macrophage.

2.8 Susceptibility of promastigote parasites to anti-leishmanial drugs and nitric oxide donors

The effect of treatment with different drugs and nitric oxide donors (SIN/SNAP) on the survival of WT and MIL-R/PMM-R *L. donovani* promastigote was determined using a resazurin assay. This assay measures the metabolic activity in cells. Mitochondria in active cells reduce the blue coloured resazurin (7-Hydroxy-3H-

phenoxazin-3-one 10-oxide) to resorufin, a pink/red coloured fluorescent product. The amount of red product produced is determined by measuring fluorescence of the samples at 550-590 wavelengths. The relative fluorescence of control and drug treated samples is then used to indicate the cytotoxicity of a sample. One hundred µL of complete HOMEM (controls) or serial dilution of the test compounds was added to the appropriate wells of a 96-well tissue culture plate. One hundred µl, *L. donovani* promastigotes ($5x10^6$ parasite/ml) of the appropriate type of parasite was added to wells containing medium alone (controls) or a test compound (n = 6/treatment). Plates were wrapped in aluminium foil to prevent exposure to light and incubated at 27°C for 72 hours and 20 µl of resazurin solution was added to each well. Plates were incubated as before for a further 18 hours. After incubation, the fluorescence of the samples was determined at 550-590 nm on a spectrophotometer using Softmax Pro 2.0 software. The percentage suppression in fluorescence of drug, SIN or SNAP nitric treated samples compared to mean control values were calculated using Microsoft Excel. The IC_{50} value for the test compounds was calculated using Probit analysis (Vermeersch *et al.*, 2009).

2.9 Amastigotes susceptibility studies

The efficacy of clinical drugs or novel compounds on the survival of WT and MIL-R/PMM-R *L. donovani* strains as well as *L. major, L. mexicana* and *L. donovani* luciferase-expressing parasites was determined *in vitro* using infected macrophages to ensure that compounds were tested against the clinically relevant amastigote stage.

On day zero, 0.5 x 10^5 macrophages per well in complete RPMI were added to the appropriate wells of a 24-well, containing a circular glass coverslip, or 96-well well plate. The final volume of each well was made up to 200 µl or 100 µl respectively with complete RPMI and plates incubated overnight at 37°C in an atmosphere of 5% CO_2; 95% air to allow macrophages to adhere. Late stage promastigotes parasites of the appropriate strain were harvested from a culture, resuspended in complete RPMI1640 medium, and the number of promastigotes/ml determined. The appropriate numbers of parasites were added to the necessary wells of each plate on day 1 at a host cell: parasite ratio of 1:40. Plates were then incubated as before to allow parasites to invade macrophages. On day 2, medium from each well was replaced in order to remove unattached parasites. Then 0.2 ml (96 well-plate) or 0.5 ml (24-well plate) complete RPMI 1640 medium (controls) or a cytotoxic compound (i.e. drug, SIN or SNAP solution) or an immunostimulant (i.e. IFN-γ 50 unit /ml: LPS 50 ng/ml or IFN-γ 100 unit /ml: LPS 100 ng/ml) was added to appropriate wells. The plate was incubated as before for a further 72 hours. The percentage of infected macrophages and the number of parasites/infected macrophage were determined as described in above. For IFN-γ:LPS treatment studies, 200 µl of cell supernatant from control and treated cells was transferred into 96 well plates and stored at -20°C to allow the nitrite and cytokine levels to be determined at a later date.

2.10 Determination of nitrite levels

Nitric oxide levels in the supernatant of *in vitro* assays were determined by Greiss assay. To determine nitrite levels of samples, 50 µl of supernatant was added to a 96 well plate Standards were prepared in two rows of the plate using serial dilutions

of sodium nitrite starting at 100 µM. To all wells, 50µl of Griess reagent (1:1 v/v mixture of 2% (w/v) sulphanilamide in 5% (v/v) orthophosphoric acid: 0.2% (w/v) naphthylene diamide hydrogen chloride) was added. After an incubation period of 5 minutes, the plate was read at 540 nm using a spectrophotometer and analysed with Softmax Pro 2.0 software. A standard curve was produced (correlation coefficient > 0.97) and used to determine nitrite levels.

2.11. Determination of cytokine levels in samples

Levels of cytokines in cell supernatants were determined by ELISA assay using anti-mouse cytokine and antibodies cytokine standards. A 96 well ELISA plate was coated with 50 µl/well of the appropriate rat anti-mouse anti-cytokine antibody, 2 µg/ml in coating buffer (PBS pH 9). Anti-cytokine antibodies used were; IL-10, IL-12, IL-6, IL-4, IL-5, TNFα and IFNγ. Plates were incubated overnight at 4°C, washed three times in wash buffer (PBS pH 7.4 containing 0.05% v/v tween-20) and then blocked using 150 µl PBS pH 7.4 containing 10% v/v FCS and incubated for 1 hour at 37°C. Plates were washed as previously described and 10 µl of cytokine standard (serially diluted in 10% v/v FCS in PBS pH 7.4 from a starting concentration of 20 ng/ml) or cell supernatant was added to the appropriate wells and incubated for 2 hours at 37°C. Plates were washed again before adding 100 µl of the appropriate rat anti-mouse biotin anti-cytokine antibody (1 µg/ml, 10% v/v FCS in PBS pH 7.4) to the appropriate well and incubated for 1 hour at 37°C. After washing, streptavidin alkaline phosphate conjugate (100 µl, 1:4000 in 10% v/v FCS in PBS pH 7.4) was added to the appropriate wells and the plate was incubated for an hour at 37°C. A final wash was performed and 100 µl of p-nitrophenyl phosphate disodium salt

hexahydrate substrate (1 mg/ml in glycine buffer [0.1 M glycerin, 2 mM magnesium dichloride, 1 mM Zinc chloride, pH 10.4]) was added to the appropriate wells. The plate was incubated for 20-60 minutes at room temperature, in the dark before measuring the absorbance of samples at 405 nm. The amount of cytokine (ng/ml) in cell supernatant was determined using a standard curve plotted from data obtained from standard samples ran on the same plate. In each case, linear regression analysis of the standards gave a correlation coefficient of > 0.97. The mean cytokine production (ng/ml ± SE) for each treatment was determined.

2.11. *In vitro* activity of MGBs in infected macrophages

Bone marrow macrophages (0.5 x 10^5/well) were added to the appropriate wells of a black 96 well plate as described previously in section 2.9. Wells were made to a final volume of 200 µl with complete RPMI and incubated at 37°C for 24 hours in an atmosphere of 5% CO_2:95% air. Late stage luciferase expressing promastigotes were added to macrophage wells at a ratio of 20:1 (parasites:macrophages) and the plate was incubated as before for 24 hours. Media from wells was removed and replaced with 200 µl of the appropriate MGB compound or control drug at a concentration of 12 µM (diluted in complete RPMI, DMSO < 1%) and incubated for 72 hours. To measure activity of the compounds, media was removed from all wells and replaced with 200 µl luciferin solution (150 µg/ml complete RPMI 1640) and the bioluminescence (BLI) emitted per well was measured (total flux/sec) using the IVIS® imaging system (IVIS® Spectrum System and Living Image® Version 4.1 software Caliper Life Sciences, Runcorn, UK). Activity of compounds was measured in percentage suppression compared to control (untreated wells). IC_{50} values were

determined by incubating luciferase parasite infected macrophages as described above over a concentration gradient of appropriate drug.

2.11. *In vivo* activity of MGBs against luciferase-expressing amastigotes

MGB-3-NO was assessed for its *in vivo* efficacy against *L. donovani* infecting BALB/c mice. On day 0, mice (n = 5) were infected with $1x10^7$ *L. donovani* LV82 amastigotes by intravenous injection into the tail vain. Mice were treated intravenously on day 7 using the following dose regimen; Control (2% DMSO), Empty-NIVs (1.5 mmol/kg NIVs, 2% v/v DMSO), MGB-3-NO solution (50 mg/kg MGB-3-NO, 2% v/v DMSO), MGB-3-NO-NIVs-150mM (50 mg/kg MGB-3-NO, 1.5 mmol/kg NIVs, 2% v/v DMSO), MGB-3-NO-NIVs-30mM (10 mg/kg MGB-3-NO, 0.3 mmol/kg NIVs, 2% v/v DMSO). Mice were sacrificed on day 14 and the parasite burdens assessed by number of macrophages infected with amastigotes and weighing affected organs.

2.12 Isolation of genomic DNA from *L. donovani*

A 5 ml culture of *Leishmania* promastigotes at day 4 of culture was pelleted by centrifugation at 2700 x g for 5 minutes and supernatant removed. The remaining pellet was resuspended in 1ml of PBS (pH 7.4) and washed twice in 1ml PBS before resuspension in 1 ml PBS (pH 7.4). Genomic DNA was prepared using the "Purification of Total DNA from Animal Blood or Cells (Spin-column)" protocol as part of the DNeasy Blood & Tissue kit (Qiagen, Crawley, UK). DNA was quantified using 1µl of DNA on a NanoDrop 2000 spectrophotometer (Thermo Scientific).

2.13 Isolation of RNA from *L. donovani*

Leishmania promastigotes at day 4 of growth were harvested as above (2.11) and resuspended in 1 ml PBS (pH7.4). RNA was isolated by following the "Purification of Total RNA from Animal Cells Using Spin Technology" of the RNeasy Mini Kit (Qiagen, Crawley UK). RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

2.14 Polymerase Chain Reaction (PCR)

Genomic DNA (gDNA) from *L. donovani* promastigotes was isolated as described in section 2.11 and used to amplify the either *L. donovani*; alpha-tubulin, LdMT or LdROS genes. Oligonucleotides used to isolate these genes were purchased from Eurofins MWG Operon (Ebersberg, Germany) and are detailed in Table 2.1. MyTaq DNA Polymerase (Bioline Reagents Ltd, London, UK) was used for all PCR reactions. Each PCR reaction contained 2 µl MyTaq™ enzyme, 10 µl MyTaq™ 5x Reaction Buffer, 25 pmol each of forward and reverse primers and 1 µg of template DNA made to a final volume of 30 µl, using molecular water. For each reaction, annealing temperature and elongation time were optimised. Reactions were performed on the Techne TC-3000 Thermo cycler (Bibby Scientific, Staffordshire, UK). PCR conditions were as follows: denaturation at 94˚C for 3 minutes, 35 cycles of 94˚C for 30 seconds, annealing 61˚C for 45 seconds and extension at 72˚C for 1 minute. Final extension was carried out at 72˚C for 10 minutes. PCR products were run on a 2% Agarose, 0.00003% v/v ethidium bromide gel.

Table 2.1 Primer sequences used to amplify LdMT or alpha-tubulin gene sequences

LdMT	Forward primer	5'- CAAGTGCCTTTCCACCAGAATC-3'
	Reverse primer	5'-CTCACCTTTTTGAACTCCAAC AGG-3'
Alpha Tubulin	Forward primer	5'-AGCTGTCCGTCGCGGACATCA CGAACTCGGTGTTT-3'
	Reverse primer	5'-CGAACTGAATTGTGCGCTTCGTCT TGATCGTCGCAAT-3'

2.15 Agarose gel electrophoresis

DNA and RNA were separated by gel electrophoresis using 0.8% w/v agarose gels containing 0.00003% ethidium bromide. Agarose was prepared using Tris-borate-EDTA (TBE) pH 8.0 consisting of 0.09 M Tris base, 0.09M H_3BO_3 and 2M EDTA. DNA loading buffer containing 0.5 x TBE buffer, 0.1 M EDTA pH 8.0, 0.1% w/v Bromophenol blue, 0.1% w/v xylene cyanol and 50% v/v glycerol was added to DNA samples at a 1:10 ratio, loading buffer:DNA. Samples were loaded on to gels and run at 120 volts for approximately an hour. A UV transilluminator was used to visualise DNA or RNA intercalated with the ethidium bromide.

2.16 cDNA Synthesis

AffinityScript™ Multiple Temperature Reverse Transcriptase was used to synthesise cDNA from *L. donovani* RNA. A total of 2 µg of RNA, 1 µl of Random Primers and molecular water was made to a final volume of 15 µl and incubated at 65°C for 5 minutes. Samples were then left at room temperature for 10 minutes before adding 2 µl of 10X Affinity script buffer, 2µl of 100mM DDT, 0.8 µl of 100mM

deoxynucleotide triphosphate (dNTP) mix and 1 µl of RT enzyme to a final volume of 20 µl. Samples were then incubated at 25°C for 10 minutes followed by 55°C for 1 hour and finally 70°C for 15 minutes.

2.17 Quantitative real time PCR (qRT-PCR)

All qRT-PCR reactions were carried out on the Stratagene Mx3000p Real Time PCR thermo cycler (Stratagene, Agilent Technologies UK Limited). Each qRT-PCR reactions contained 6.25 µl SYBRgreen, 1 µl template cDNA (prepared in section 2.15), 25 pmol of forward and reverse primers and made to a final volume of 12.5 µl using molecular grade water. For negative control samples, molecular water replaced the template cDNA. Conditions were as follows: Denaturation at 95˚C for 10 minutes, 40 cycles of 95˚C for 30 seconds, annealing 63˚C for 45 seconds and extension at 72˚C for 1 minute and a final cycle of 95°C for 30 seconds and 55˚C for 30 seconds. All samples were carried out in duplicate. C_T values for samples was calculated using Stratagene MX3000 Pro QPCR software (2007 Stratagene© version 4.10) software. Gene expression was calculated using the $^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001). The housekeeping gene α-tubulin was used as a comparison for expression levels.

2.18 Metabolome/lipidome extraction

Extraction of the metabolome or lipidome of *L. donovani*, as described below, was carried out using day 4 promastigote stage of the lifecycle. The metabolism of cells was halted by quenching cultures to 0°C using and ethanol-dry ice bath. Four

aliquots per strain of 4 x 10^7 cells were pipetted in to pre-chilled Eppendorf and kept at 0°C throughout the extraction.

Aliquots were pelleted by centrifugation at 2700 x g for 5 minutes and the supernatant was aliquoted in to separate Eppendorf tubes and stored at -20°C for later analysis. Cells pellets were then washed by re-suspending them in 1 mL of PBS, pre-chilled to 0^oC. They were then centrifuged at 2700 x g for 5 minutes and the supernatant was removed. Excess PBS was removed by a final centrifugation step of at 2700 x g for 10 minutes. After washing, cells were re-suspended in 200 µL of chloroform:methanol:water, 20:60:20 v/v ratio, for metabolome extractions and 50:50, methanol:chloroform v/v ratio, for lipidome extractions. These mixtures were then shaken for 1 hour at 1,400 rpm, 0^oC in a thermomixer before being centrifuged at 2700 x g for 5 minutes at 0^oC. The resulting supernatant was removed and transferred to a mass spectrometry vial fitted with a low-volume insert. These vials were then stored at -70^oC until analysed.

2.19 Miltefosine uptake studies

The uptake of MIL in to WT and MIL-R promastigote cells was quantified using LC-MS. Briefly, MIL was added to 5 ml cultures of promastigotes at day 4 growth to a final concentration of 7 µM MIL. Flasks were quenched at time 0, 30, 60 and 120 minutes post exposure to MIL and 4 x 10^7 parasites were aliquoted in to pre-chilled eppindorf tubes and stored on ice. Lipidomic extraction was then carried out as described above in 2.18. To accurately quantify the concentration of MIL in samples, a calibration curve was produced by spiking extraction solvent samples

with MIL standards at 0.24, 0.72, 2.4, 7.2 and 14.4 µM MIL. Linear regression was used to fit a standard curve for the area under the curve of the MIL peak from the standards. Only calibration curves of correlation coefficient > 0.97 were used in experiments.

2.20 High pressure liquid chromatography – mass spectrometry (HPLC-MS)

All samples were analysed on the same instrumentation and under the same conditions. The HPLC system used was a Dionex Ultimate 3000 (Thermo Fisher Scientific Inc., Waltham, USA) composed of a pump, autosampler and column compartment. A SeQuant ZIC®-pHILIC (Merck) column was used to run metabolomic samples. A Silica gel column (150 mm x 3 mm x 3 µm, HiChrom, Reading, UK) was used for lipidomic extracts. The columns were connected to a Thermo Scientific Exactive Mass Spectrometer running in positive/negative scanning mode with the following parameter settings:

For metabolomic samples, two solvents (A) Acetonitrile and (B) Ammonium carbonate (20 mM, pH 9.2) in water were used. For lipidomic samples, two solvents (A) 20% v/v isopropyl alcohol (IPA) in acetonitrile and (B) 20% v/w IPA in ammonium formate (20mM) were used. Elution was achieved using the following gradient at 0.3 mL/min: 0-1 min 8% B, 5 min 9% B, 10 min 20% B, 16 min 25% B, 23 min 35% B, 26-40 min 8% B.

Table 2.2 LC-MS Scan parameters

Scan Parameters	Setting
Scan range	100 – 1200
Resolution	50,000
Microscans	3
Lock masses (positive/negative)	195.0877 / 265.1479
ACG target	1,000,000
Maximum inject time	250 ms

Table 1.3 LC-MS ESI source parameters

ESI Source Parameters	Setting
Sheath gas flow rate	50
Aux gas flow rate	17
Sweep gas flow rate	0
Spray voltage (positive/negative) (|kV|)	4500 / 4000
Capillary temperature (^{o}C)	275
Capillary voltage (positive/negative) (V)	28 / -30
Tube lens voltage (positive/negative) (V)	95 / -110
Skimmer voltage (positive/negative) (V)	21 / -15

2.21 Data Analysis of Metabolomic Samples

Raw data files produced by the Exactive mass spectrometer were converted to mxXML format and centroided using MSConvert (Proteowizard, http://proteowizard.sourceforge.net/downloads.shtml). Individual peaks were picked based on non-linear retention time alignment, feature detection and feature aligningment using the centwave method from XCMS (https://metlin.scripps.edu/xcms/). These peaks were converted to .peakML files using MzMatch.R (http://mzmatch.sourceforge.net/ index.php). MzMatch.R was then used for peak extraction, filtering, normalisation, group filtering and gap-filling of peaks. This produced a single file in .text format containing peak groups with the same mass/charge (m/z) values from the different biological replicates. Further filtering and individual metabolite identification was performed using the Excel interface IDEOM (Creek *et al.*, 2012). The mass chromatograms were putatively identified in IDEOM by matching the recorded mass to the accurate mass of metabolites within LeishDB. The threshold for identification was < 3 parts per million (ppm). For lipidomic data, the .txt files were read using Microsoft Excel and analysed manually using Xcalibur™ (Thermo Scientific). Briefly, peaks were separated in to classes of lipids based on their retention time. Individually, the measured mass of each peak was input in to the Elemental Composition tab in Excalibur to identify the chemical formula. A threshold of <3 Delta milli mass units (mmu) between the measured and accurate mass of the given formula was used. The number of carbons and double bonds in the side chains of a given lipid was

calculated using the Lipid Predictor 3000 Excel interface developed by Dr Gavin Blackburn, University of Strathclyde.

Lipids nomenclature in this study is as follows; X(y:z) where x = lipid class, y= total carbons in sidechain and z = number of double bonds present in sidechain. For example PC (24:0) will be a PC with the standard PC head group of $C_8H_{18}O_8NP$, two side chains that will contain a total of 24 carbons between them and 0, c-c double bonds. The exact number of carbons per sidechain, or the position of any double bonds, cannot be accurately predicted without fragmentation.

2.22 Statistical Analysis

Data was first tested for normality using a Kolmogorov–Smirnov test normality test. Metabolomic and lipidomic data comparing WT vs WT or WT vs Drug-Resistant were analysed using two tailed, heteroscedastic, student's t-test using or a Mann Whitney U-test. *In vitro* data that did not have a normal distribution was analysed using a Mann-Whitney U test for comparing two treatments or a Kruskal Wallis test followed by Dunns ad-hoc test for testing between three or more treatments. P-values were corrected for multiple testing using the Benjamini-Hochberg method using R. Statistical software packages used included; SSPSS (IBM, New York, USA), Minitab 15 (Minitab Inc., Pennsylvania, USA), Origin Pro 9.3 (OriginLab Corporation, Northampton, USA), GraphPad Prism 6.05 (GraphPad, California, USA), Statview v5.0.1 (Scientific Computing, North Carolina, USA) or Microsoft Excel 2010 (Microsoft, Redmond, USA). P values below 0.05 were considered significant.

Chapter 3. Selecting Drug Resistance and Cloning Drug Resistant Parasites

3.1 Introduction

Resistance to Sb has rendered drugs such as SSG obsolete in endemic areas such as the ISC. Consequently, alternatives such as L-AmB MIL and a combination of MIL with PMM are being used to treat patients. With few other alternatives, resistance to these drugs would represent a major problem in the treatment of *Leishmania*. If or how this inherent susceptibility to Sb affects the development or mechanisms of acquired MIL or PMM resistance is as yet unknown. In the course of this study, we aimed to compare the inherent susceptibility to Sb of *L. donovani* isolates from Nepal and the impact of inducing resistance to MIL and PMM on these isolates. To properly study the effects of the development of drug resistance, wild type promastigote parasites from three different backgrounds of Sb sensitivity were made resistant, separately, to MIL and PMM. Populations of resistant parasites contain a genetically heterogeneous mix of parasites that would make elucidating a genetic and metabolomic mechanism of resistance impossible. For this reason, populations of drug resistant parasites were cloned from one single cell to produce a genetically identical population from which all further studies used.

Therefore the main objectives of this study were:

1) Produce populations of *L. donovani* that are resistant to i) MIL and ii) PMM

2) Clone MIL and PMM resistant lines of parasites to create clonal populations of parasites

3.2. Results

Drug resistance in promastigote parasites was selected for via exposure to a stepwise increase in drug concentration over a number of weeks. To achieve this, promastigotes were cultured with a sub lethal concentration of drug until growth rates matched that of their corresponding WT promastigotes. The drug concentration in growth media was then increased likewise until maximum achievable concentrations were achieved.

3.2.1 Selecting miltefosine resistant parasites

All work to select for MIL resistance and testing of drug susceptibility at intermediate stages was carried out by Dr. Julien Lonchamp at Strathclyde University. The WT isolates selected for resistance to MIL were Sb-S (Sb sensitive) WT and Sb-R (Sb resistant) WT. The maximum concentration of MIL used to culture promastigotes was 74 μM as above this concentration, levels of MIL were observed to be toxic to host macrophages used in amastigote studies. This was observed by membrane deterioration in macrophages when stained with Giemsa stain after 72 hours incubation. It took a total of 31 weeks of incremental drug pressure to adapt WT strains to the highest concentration of MIL and both WT strains adapted to MIL at similar rates. During induction of resistance, the susceptibility of intracellular amastigotes to MIL was tested to determine if resistance in the promastigote stage conferred resistance in the amastigote stage. In both Sb-S and Sb-R strains, the IC_{50} of amastigotes to MIL was not observed to increase in parallel when promastigotes

tolerated 3 and 6μM MIL (Table 3.1). Only when promastigotes tolerated 12μM MIL was a tolerance in the intracellular amastigote stage observed.

Table 3.1. IC_{50} and IC_{90} values of intracellular amastigote Wild type and MIL adapted parasites.

MIL tolerated by promastigote (μM)	Sb-S amastigote		Sb-R amastigote		
	MIL IC_{50}	MIL IC_{90}	MIL IC_{50}	MIL IC_{90}	n
0 (WT)	3.1 ± 1.3	4.6	3.1 ± 1.4	6.1 ± 0.7	6
3	1.2	5.6	1	3.4	1
6	2	4.6	2	6.0	1
12	14	41.8	9	47.0	1
49	47	181.0	33	103.8	1
74	44.3 ± 13.2	162 ± 9.5	35 ± 7.9	158 ± 10.7	3

The final increase in MIL concentration in promastigote culture from 49μM to 74μM did not yield an analogous increase in MIL IC_{50} in the intracellular amastigote stage. Nonetheless, selected resistance to MIL in promastigotes clearly conferred resistance to MIL in the intracellular amastigote stage. The IC_{50} of Sb-S MIL-R amastigotes was 44 μM, corresponding to a 15 times increase in tolerance to MIL. Likewise, Sb-R MIL-R amastigotes (IC_{50} 35 μM) were 13 times more resistant compared to their original WT counterparts (3 μM). Increasing the MIL concentration to select MIL-R promastigotes from 49.2 to 74 μM did not yield a corresponding increase in amastigote parasites (Table 3.1).

3.2.2 Paromomycin selection

As well as Sb-R and Sb-S WT isolates, an additional isolate, Sb-I (Sb intermediate resistant) strain was selected for resistance to PMM. For all three lines, 97 μM PMM was the highest achievable drug concentration tolerated in media as above this,

promastigotes did not replicate WT growth. It took 26 weeks of passaging with increasing drug concentration to reach the maximum of 97 µM PMM. Growth rates of WT and PMM-R clones are shown in Figure 3.1.

Unexpectedly, Sb-S WT was found to have an inherent resistance to PMM at both the promastigote and intracellular amastigote stages. Nevertheless, Sb-S was exposed to increasing PMM concentrations and cloned under the same conditions as Sb-I and Sb-R strains being selected for resistance to PMM. Due to time constrains, the intermediate resistant promastigotes between 0 and 97 µM PMM were not analysed for their susceptibility to the drug. Both Sb-I and Sb-S parasites adapted to PMM at similar rates.

3.2.3 Cloning miltefosine resistant parasites

Resistant parasites were subsequently cloned using two different techniques; a dilution method and a hanging drop method. The dilution method failed to achieve cloned parasites capable of sustained growth and so did not yield any viable populations. Ten clones per parental population were isolated using the hanging drop method. Not all the clones proliferated to produce populations, however, at least three clones per drug resistant population were produced, allowing one suitable clone to be selected per population. Sb-S MIL-R produced 4 clonal populations while Sb-R MIL-R parental population produced 3 clones.

To confirm that cloned parasites had retained their resistance to MIL, clonal populations were tested for their susceptibility to MIL at the promastigote stage. In all cases, the IC_{50} of the cloned population had decreased compared to its parental

population (Tables 3.1 and 3.2). One clone from each of the Sb-R and Sb-S was selected based on both the IC_{50} of promastigotes and how well clones grew under continual culture with 74 μM MIL. Sb-S MIL-R clone number 8 was selected and for Sb-R MIL-R, clone number 9 was selected. From this point, Sb-S MIL-R clone 8 is referred to as Sb-S MIL-R and Sb-R MIL-R, clone 9 as Sb-R MIL-R.

The susceptibility to MIL of both clones was then tested to ensure that promastigotes had maintained their resistance to MIL in the intracellular amastigote stage. The IC_{50} to MIL of Sb-S MIL-R was again less than that of their parent populations, indicating that selection of resistance resulted in a mixed population of parasites with variable susceptibilities to MIL (Table 3.2). The IC_{50} value of Sb-S MIL-R parent and Sb-S MIL-R parasites were similar. Removal of MIL pressure for 4 weeks of culture did not alter the MIL susceptibility of Sb-S MIL-R cloned population. Conversely, cloned Sb-R MIL-R had a higher IC_{50} value compared to its parent population (Table 3.2). Culturing the Sb-R MIL-R cloned parasites in the absence of MIL resulted in a small decrease in MIL susceptibility to that similar to the parental range (data not shown) but tolerance to MIL was maintained nonetheless. Selection of MIL resistance did not impact upon the susceptibility to SSG and both MIL-R cloned parasites had a similar dose-response to SSG treatment compared to WT (data not shown).

Table 3.2. IC_{50} of Sb-S and Sb-R WT and selected MIL resistant clone.

		MIL IC_{50} (µM)	
		Promastigote	**Amastigote**
Sb-S	WT	13 ± 1	3 ± 1
	MIL-R parent	371 ± 15	44 ± 13
	MIL-R clone	254 ± 15	46 ± 18
Sb-R	WT	6 ± 1	3 ± 1
	MIL-R parent	357 ± 3	35 ± 8
	MIL-R clone	294 ± 6	53 ± 1

3.2.4 Cloning paromomycin resistant parasites

Sb-S, Sb-I and Sb-R PMM-R parental populations produced 9, 4 and 1 viable clonal populations respectively from their original 10 cloned promastigotes. All clones were screened for their susceptibility to PMM at the promastigote stage. One clone per strain was chosen based on susceptibility to PMM and growth *in vitro*. Clones selected were; Sb-S PMM-R clone 3 now referred to as Sb-S PMM-R, Sb-I PMM-R clone 23 (Sb-I PMM-R) and Sb-R PMM-R clone 63 (Sb-R PMM-R). The clone of the inherently PMM resistant, Sb-S WT selected was found to have a small decrease in resistance to PMM in both promastigote and intracellular amastigote stages. Both Sb-I and Sb-R PMM-R clones retained significant resistance to PMM compared to the original WT populations at promastigote and amastigote stages ($p < 0.05$, Table 3.3). Sb-I PMM-R promastigotes were nearly nine times more resistant to PMM than WT, 481 µM compared to 56µM respectively. The intracellular amastigote IC_{50} of 195 µM in Sb-I PMM-R was over three times greater than that of it Sb-I WT IC_{50}, 56 µM. Likewise, Sb-R PMM-R amastigotes (IC_{50} 165 µM) were two and a half times

more resistant to PMM than Sb-R WT (67 µM). A sevenfold increase in promastigote PMM resistance from 65 µM to 455 µM IC_{50} between Sb-R WT and PMM-R promastigotes was observed.

Table 3.3. IC_{50} of Sb-S, Sb-I and Sb-R WT and selected PMM resistant clones. *p < 0.05 for PMM-R the corresponding WT.

		PMM IC_{50} (µM)	
		Promastigote	**Amastigote**
Sb-S	WT	354 ±4	166 ±11
	PMM-R	305 ±27	155 ±26
Sb-I	WT	56 ±22	56 ±18
	PMM-R	481 ±84*	195 ±12*
Sb-R	WT	65 ±2	67 ±1
	PMM-R	455 ±6*	165 ±20*

3.2.5 Assessment of growth of drug resistant parasites

Growth curves of the three WT isolates and their respective clones were analysed to ensure drug resistant clonal populations grew as normal. Promastigotes from culture were sampled daily for seven days and the total number of cells/ml was recorded (Figure 3.1). In culture *Leishmania* replicate once every 6-8 hours and growth follows a sigmoidal shape when plotted on a graph. Growth is characterised by three main phases; mid-log, late-log and stationary phases of growth. All of our WT and drug resistant clones followed comparable patterns of growth. All cell lines transitioned from mid to log-phase at around day 3 and entered the stationary phase between days 6 and 7. Likewise, all lines achieved final densities of between 3.5 and 4.5 $x10^7$ cells/ml by day 7. There was no apparent, significant difference in

the rate of growth or the final density reached between any WT promastigotes and their drug resistant variants.

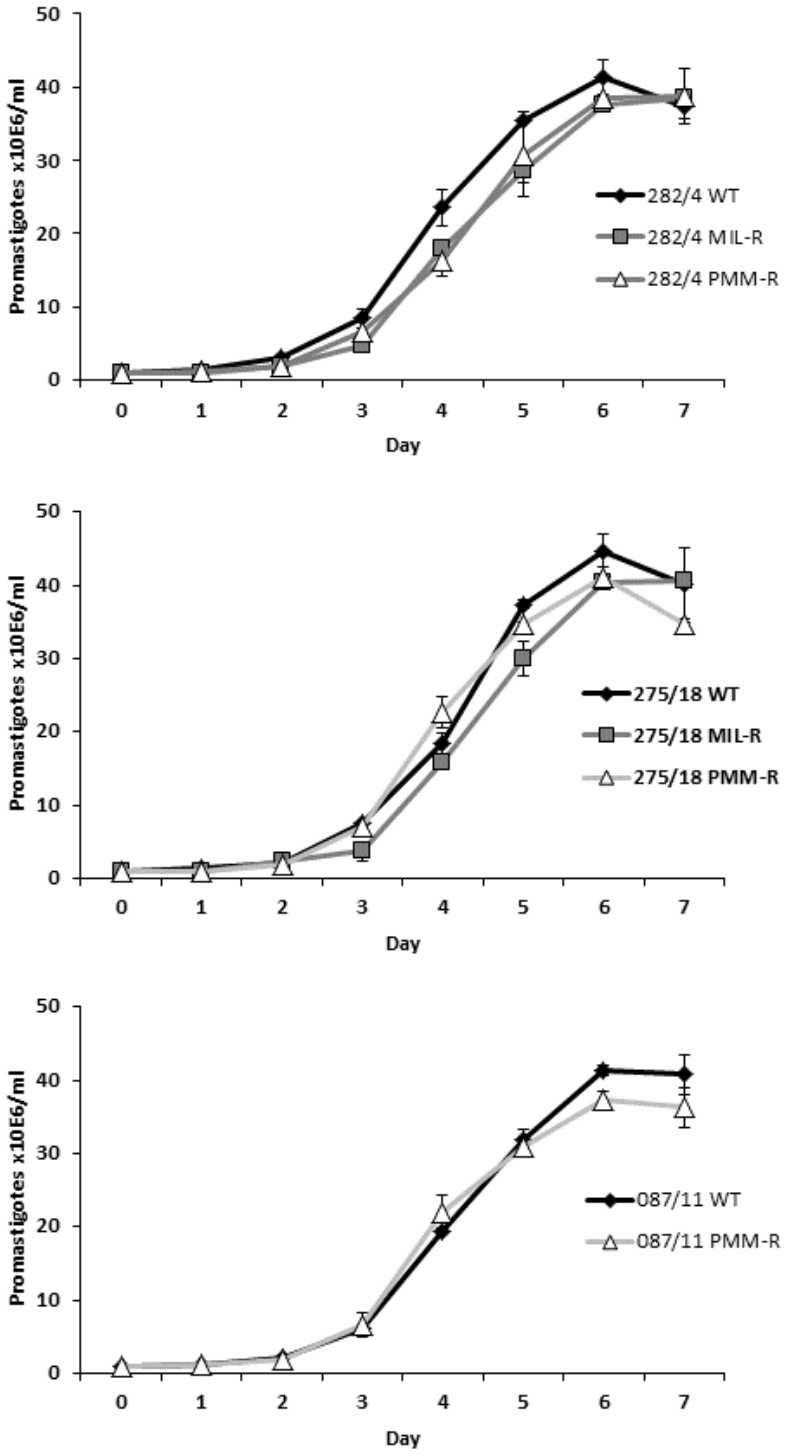


Figure 3.1. Growth curves of WT, MIL-R and PMM-R promastigote parasites. Promastigotes cultures were seeded at $1x10^6$ cells/ml and counted daily for 7 days to assess growth. No significant difference in the growth rate of any drug resistant clone compared to WT was found.

3.3 Discussion

We successfully performed a stepwise selection of MIL and PMM resistance in WT Sb-S and Sb-R strains derived from clinical isolates from Nepal. Additionally, PMM resistance was also successfully selected for in Sb-I WT promastigotes. These data show that resistance to both MIL and PMM can be rapidly selected for in promastigotes *in vitro* and this drug resistance is translated in to the intracellular amastigote stage. For MIL resistance, we generated resistant lines with 28 to 57 fold increased tolerance to MIL in the promastigote stage. For intracellular amastigotes the increase in tolerance was around 13 to 15 times compared to WT. For PMM, the situation was complicated by Sb-S WT having a natural resistance to PMM without (known) prior exposure to the drug. In the two WT lines that were not already resistant to PMM we generated resistant lines with a 7 to 8 fold decrease in promastigote susceptibility and a 2 to 3 fold decrease in intracellular amastigote susceptibility.

Tolerance to MIL was tracked at intermediate stages of the selection process and a significant increase of the IC_{50} for the intracellular amastigote stage was only present at concentrations above 12 µM MIL. Pharmacokinetic studies on MIL suggest that blood levels above this concentration are likely achieved in a normal treatment regimen. Treatment with MIL at 100 mg/kg for 28 days typically resulted in a maximal median plasma concentration of 70 µg/ml (172 µM) on day 23 (Dorlo *et al.*, 2012). This is significantly greater than the 12 µM needed to create a decrease in susceptibility of intracellular amastigotes seen in this study, indicating that *in vivo* levels of MIL during treatment could be above the threshold at which

resistance can start to occur. Despite this, we observed considerable toxicity in macrophages treated with 148 µM MIL, indicating that this concentration would be cytotoxic if it is maintained *in vivo* after dosing. It is therefore likely that intracellular levels of MIL are much lower than 172 µM. Moreover, intracellular amastigotes in the PV are likely to be exposed to lesser concentrations of MIL than those in the blood circulation and have limited exposure to high concentrations of MIL. It is possible that this is an explanation for why it has not been possible to isolate parasites with a decreased susceptibility to MIL recovered from MIL relapse patients in India (Rijal *et al.*, 2013).

Cloned parasites derived from parental lines of drug resistant parasites maintained resistance to MIL or PMM, albeit at varying degrees. This supports the polyclonal nature of a resistant population that has individual cells with varying degrees of susceptibility and justifies the choice to clone drug resistant populations prior to genomic and metabolomic studies in order to create a uniform population of parasites that would help produce consistent results (Coelho *et al.*, 2012b).

The background Sb sensitivity of parasites appeared to have no influence on the rate at which resistance to either drug was selected for. Both the Sb-S and Sb-R isolates reached comparable levels of MIL resistance over the same period of time. Surprisingly, Sb-S WT was found to be inherently resistant to PMM however this does not appear to be directly linked to the Sb susceptibility as the intermediate Sb resistant Sb-I adapted to PMM at the same rate as the Sb-R. Moreover, several other Sb-S *L. donovani* clinical isolates were tested for their susceptibility to PMM

with no other cases of inherent PMM resistance found. All strains used were clinical isolates, obtained from Nepalese patients. As far as is known, the patient from which this isolate was isolated from had no previous contact with PMM. Likewise, the isolated Sb-S strain had never been subjected to PMM in the laboratory before its susceptibility was tested. This suggests that either; a) naturally occurring, PMM resistant populations of *L. donovani* may be present in the wild or b) *L. donovani* parasites that have been exposed to PMM can easily become resistant and are now present in the wild. Either way, this has implications for the suitability for the drug as a long term treatment in the fight against leishmaniasis. It has been documented as far back as 1995 that clinical isolates of *L. donovani* have varying susceptibilities to PMM (Neal *et al* 1995). PMM was only registered for use against leishmaniasis in 2006 (Sinha *et al.*, 2011). Any resistance as a result of exposure to the drug would therefore have built up extremely quickly. If this is the case, it could imply that resistance in the field would occur easily and the shelf life for PMM as an anti-leishmanial would be short lived.

Resistance to PMM and MIL can be selected for relatively easily in the laboratory and has been reported in several studies previously. The majority of studies have utilised drug resistant strains generated *in vitro* using promastigotes and a step wise increase in drug concentration, the same technique used in this study. Seifert generated a MIL-R strain in 2003 using such a method and MHOM/ET/67/L82 parasites (Seifert *et al.* 2003). These parasites were since used in several other studies designed to elucidate the impact of acquired drug resistance (Perez-Victoria *et al.* 2003, Rakatomanga *et al.* 2007, Garcia-Hernandez *et al.* 2012). Interestingly,

the maximum concentration of MIL used in selection by Seifert was 40 µM MIL. Above 50 µM, Seifert reported non-specific detergent effects of MIL. In this study however we were able to culture parasites with a maximal concentration of 74 µM MIL without any noticeable detriment as parasites grew as WT. This may be due to either the strain of parasite used or the length of time parasites were cultured between each incremental increase in drug pressure. Seifert reported MIL-R parasites were 15 times more resistant compared to their WT whereas our MIL-R promastigotes were up to 57 times more resistant. This difference is likely due to the fact that we were able to culture promastigotes to 74 µM compared to 40 µM MIL. Hendrickx *et al.* attempted to generate MIL-R parasites selected for using amastigotes exposed to MIL rather than promastigotes (Hendrickx *et al.*2014). Interestingly, an increase in resistance to MIL could not be identified in the amastigotes stage but was seen when amastigotes were back transformed to the promastigote stage and assessed. The same study also generated amastigotes resistant to PMM. Unlike the MIL-R parasites, PMM-R amastigotes were easily generated and identified *in vitro* when amastigotes were exposed to increasing drug pressure. The ability to produce and isolate PMM-R parasites generated using the amastigote stage had also been reported in an earlier study by the same group (Hendrickx *et al.* 2012). Similar results have been observed *in vivo* where PMM-R amastigotes can be easily generated in Syrian hamsters using treatment/relapse cycles (Hendrickx *et al.* 2015). Maarouf *et al.* have previously generated PMM-R parasites using the stepwise increase of drug pressure on the promastigote stage, similar to this study (Maarouf *et al.* 1998). Strikingly, while we achieved a maximal

culture concentration of 97 µM PMM, Maarouf achieved parasites resistant to concentrations of 800 µM PMM.

The dilution method of cloning parasites failed to yield any suitable clones. The method relies on growing up parasites from concentrations that in theory numerically do not contain any parasites. It is however an unreliable method. Although individual wells can be examined for single parasites, it is impractical on a large scale. Confirming that only one parasite is present in the relatively large volume of one well of a 96 well plate is difficult. Using the dilution method, it is impossible to confirm that any populations grown up are definitively from one single parasite.

Not only was the hanging drop method more successful at generating cloned populations, it is a more reliable method for ensuring correctly cloned populations. The micro-drop used is easier to check for the presence of only one parasite. Typically, the whole micro-drop can fit in to one field of view at x20 magnification on a microscope. Moreover, it is easier to confirm with a second observer that only one parasite is present in the sample. Prior to the method, parasites were adapted on to Tobie's blood agar, overlaid with Locke Media. The blood agar provides an environment that is more favourable for parasite growth, particularly in low numbers, compared to more standard media such as DMEM or HOMEM (Neal and Miles, 1963). It requires a ready supply of blood and is time consuming to prepare, therefore long term culture of parasites using blood agar is not viable. The presence of erythrocytes and other metabolites from the blood used in the agar also means it

is unsuitable for several assays. Despite this, we found success using it for cloning *L. donovani*. Cloning parasites using our standard HOMEM did not yield any populations.

Clones within each individual strain varied in their resistance to MIL compared to the parental population. This is again indicative that parental populations contained a heterogeneous population of parasites with varying degrees of resistance to MIL. Cloning therefore selected one of these parasites and grew a homogeneous population. Clones were selected on the basis of their high IC_{50} value and their growth from week to week. Clones showing high resistance to MIL in assays but poor growth were discarded.

Leishmania promastigotes typically grow in an S-shaped curve over the course of 7 days. All of the clones selected showed the correct growth cycles and were comparable to the growth of their WT ancestors. It therefore appears that the selected drug resistance did not detrimentally affect parasite growth or differentiation.

In conclusion, *L. donovani* promastigotes were successfully selected for resistance to both MIL and PMM and the main objectives covered were:

- Promastigotes resistant to MIL and PMM were rapidly selected *in vitro* via a stepwise increase in drug pressure over a number of weeks.
- Resistance in the promastigote stage was conferred to the intracellular amastigote stage and was stable even in the absence of drug pressure.

- Drug resistant promastigotes were cloned using the hanging drop method to enable further studies to be conducted using a genetically homogenous, drug resistant population.
- Cloned parasites differed slightly in their susceptibility to MIL or PMM compared to their drug resistant parent indicating that resistant populations contained a mixture of parasites with variable levels of resistance.

Chapter 4. Metabolomic Differences Associated With Inherent Susceptibility to Antimony

4.1 Introduction

Metabolomics can be used to take a snapshot of the chemical composition and processes within an organism at a given time to complement data acquired from genomic, transcriptomic and proteomic analyses to build a complete picture of an organism's biology (Vincent and Barrett, 2015). Using the appropriate solvents, the metabolome of an organism can be extracted and individual metabolites separated and putatively identified using LC-MS. Two basic types of metabolomic analysis can be undertaken; untargeted and target. Untargeted analyses look at the metabolome indiscriminately as one big picture whereas a targeted analysis involves a more focused approach, looking for a particular metabolite(s) or pathway of interest. More specifically, metabolomics can be employed as a tool to look at the mode of action of drugs or the mechanisms of drug resistance. Analysing the metabolome of an organism pre- and post-drug treatment provides an insight in to what chemical changes have occurred on exposure to the drug, and this is most appropriate for a drug that affects biochemical targets within an organism. Similarly, analysing the metabolome before and after inducing drug resistance can be used to identify the biochemical changes associated with acquired resistance. This has the potential to prolong the life of a drug by combining drug treatment with specific inhibitors targeting pathways responsible for drug resistance or can help influence future drug design so that new drugs target other critical pathways that have not been associated with drug resistance. For example, if resistance to a particular drug (drug A) is caused by the active efflux of the drug from the target organism/cell, co-administering the drug with a second drug (drug B) that can

inhibit the efflux of drug A from the cell should be able to circumvent the acquired resistance and prolong the clinical life of drug A. For example, the calcium channel blocker, verampamil can reverse resistance to chloroquine in the malaria causing parasite, *Plasmodium* (Martin *et al.* 1987). Likewise, conjugating the quinolone moiety of chloroquine to efflux inhibitors has been shown to increase the potency of chloroquine against *Plasmodium falciparum* (reviewed Peyton 2012). Considering the small number of drugs available to treat VL and the lack of any future chemotherapy in the pipeline, prolonging the use and preventing resistance to current chemotherapeutic options is imperative.

Antimonials, including SSG, have been used to treat VL for over 80 years. Due to increasing incidences of resistance, their role as the first line treatment of VL in India was abandoned in 2002 and replaced by L-AmB (Sundar et al., 2002b). Thirteen years later however, SSG resistant populations are still found in the ISC, implying that a resistance to SSG confers a selective advantage to survival, even in the absence of SSG treatment, or at the very least, is not detrimental to parasites (Vanaerschot *et al.*, 2014). An exact mechanism for SSG resistance is yet to be identified and it is likely that it is the result of multiple mechanisms working synergistically. Several laboratory studies have concluded that Sb-R parasites are more resistant to macrophage killing compared to Sb susceptible parasites (Carter *et al.*, 2005, Guha *et al.*, 2014, Mukhopadhyay *et al.*, 2011, Vanaerschot *et al.*, 2014). Other mechanisms such as alterations in the glycerol transporter, aquaglyceroporin 1 (AQP1) and metabolism of ornithine decarboxylase have been strongly implicated in playing a role in SSG resistance (Decuypere *et al.*, 2012).

Genomic analyses have identified that SSG resistance has in fact emerged several times via a number of independent events, highlighting that multiple, different mechanisms of resistance may exist in endemic parasite populations (Decuypere *et al.*, 2012, Downing *et al.*, 2011b, Downing *et al.*, 2011a).

Several metabolomic analyses on the backgrounds of Sb-R parasites or treatment with Sb have already been published (t'Kindt *et al.*, 2010, Berg *et al.*, 2015, Canuto *et al.*, 2012). In two of these studies, increased levels of glutathione disulphide, L-cystathione and several other metabolites involved in the oxidative response were upregulated (Canuto *et al.*, 2012, t'Kindt *et al.*, 2010).This is in agreement with much of the previously published work that implicated an increased oxidative stress response with Sb treatment (Carter *et al.*, 2006, Carter *et al.*, 2005, Vanaerschot *et al.*, 2014). Berg *et al.* sought to underpin the MOA of SSG using a metabolomic approach (Berg *et al.*, 2015). SSG resistant and susceptible parasites were analysed in the presence and absence of drug pressure in an effort to elucidate mechanism(s) responsible for antimony resistance. Large numbers of metabolites were seen to alter between each treatment group and individual strains so specific mode of action could not be identified. This would support the hypothesis that SSG has a multi factorial mechanism and affects several targets in the parasite. In another study analysing the difference between susceptible and resistant strains, t'Kindt (2014) reported that several lipids classes differed between the strains suggesting that lipid, perhaps in the plasma membrane could play a role in SSG resistance.

A need to understand how drug resistance develops is the focus of a number of studies directed at the mechanisms and implications of resistance to the commonly used anti-leishmanial drugs MIL, AmB and PMM. The majority of these studies use reference strains of *L. donovani* that have been studied extensively for several years. With a prevalence of both Sb-R and susceptible parasites existing in endemic parasite population, it is important to first understand the differences between these parasites rather than simply concentrating on laboratory strains. Reference strains do not take in to account genetic variability that occurs in the field, nor do they take into account prior adaptation to clinically used drugs in particular regions. Therefore, in this project we used two Nepalese field isolates of *L. donovani* which had characterised differences in their susceptibility to Sb. Using these, we first wanted to understand how susceptibility to Sb differed in both the metabolome and genome of the parasites before analysing if these factors altered their ability to acquire drug resistance or the mechanism which it was acquired.

Therefore the main objectives of this study were to use two wild-type (WT) clinical isolates from Nepal to:

1) Determine if differences in susceptibility to Sb is associated with specific metabolomic differences.

2) Determine if differences in susceptibility to Sb is associated with specific lipidome differences.

3) Correlate any differences in the metabolome/lipidome to observed differences in the genome of *L. donovani* field isolates.

4.2 Results

4.2.1 Metabolomic differences associated with antimonial resistance

Genomic sequencing of the Nepalese strains confirmed that the two strains are genetically very similar (Downing *et al.* 2011). The two strains were mapped closely on the phylogenetic tree (Downing *et al.* 2011) and were distinguished by only 7 differences in chromosome copy number and 127 individual SNPs. This maximises the possibility that any observed difference in the metabolomes are related to the sensitivity towards antimonials. The metabolomic backgrounds of the two WT strains were compared to assess if resistance to Sb had an impact on the metabolome and lipidome of parasites. It was important to know the base-line for each strain so that differences identified in MIL-R parasites were not simply due to initial differences between the two WT parents.

An untargeted analysis of the whole metabolomes of both Sb-S and Sb-R WT typically identified 7500 – 8000 peaks per experiment. After data processing, which included gap filling and peak picking, between 700-900 peaks were found and 400-500 of these were putatively identified as metabolites. Annotated metabolites were putatively named by IDEOM and classified based on their biochemical properties or predicted function. IDEOM assigns putative names to metabolites based on a combination of retention time, exact mass and presence in Leish DB. Every metabolomic extraction was run in tandem with selected metabolite standards to correct for retention time drift and fluctuations in running conditions i.e. room and column temperature, sample temperature, overall condition of the column and day

to day machine performance which have the potential to skew results. IDEOM assigns an ascending confidence value between 1 and 10 to each metabolite identified.

Metabolites run as standards are assigned an identification confidence value of 10. A cut off value of 7 and above was used for metabolites analysed in this study to reduce ambiguity of identified metabolites. To evaluate the efficiency of the extraction and identification procedures, the LC chromatogram and general metabolomic profile of parasites was evaluated in each experiment. Metabolites with abnormal chromatograms or significant drift between peaks of replicate samples were discarded. For each study detailed below, experiments had 4 biological replicates per experiment and at least three independent experiments were carried out in the study. Only metabolites that were consistently identified in all three independent experiments were considered. It is important to note that due to the variability in the performance of LC-MS caused by factors such as retention time drift, ion suppression, sensitivity to temperature fluctuations and column health, data sets acquired from the LC-MS at different times means that there can be some variation between experiments. For this reason, independent runs cannot be cumulatively compared with each other, however, the trend between experiments can be directly compared. For example, peak intensities for a particular metabolite can vary depending on a number of factors and is not expected to be a consistent, exact value between different experiments. For this reason ratios between peaks within the same experiment were calculated and used to compare the peak relationships between experiments.

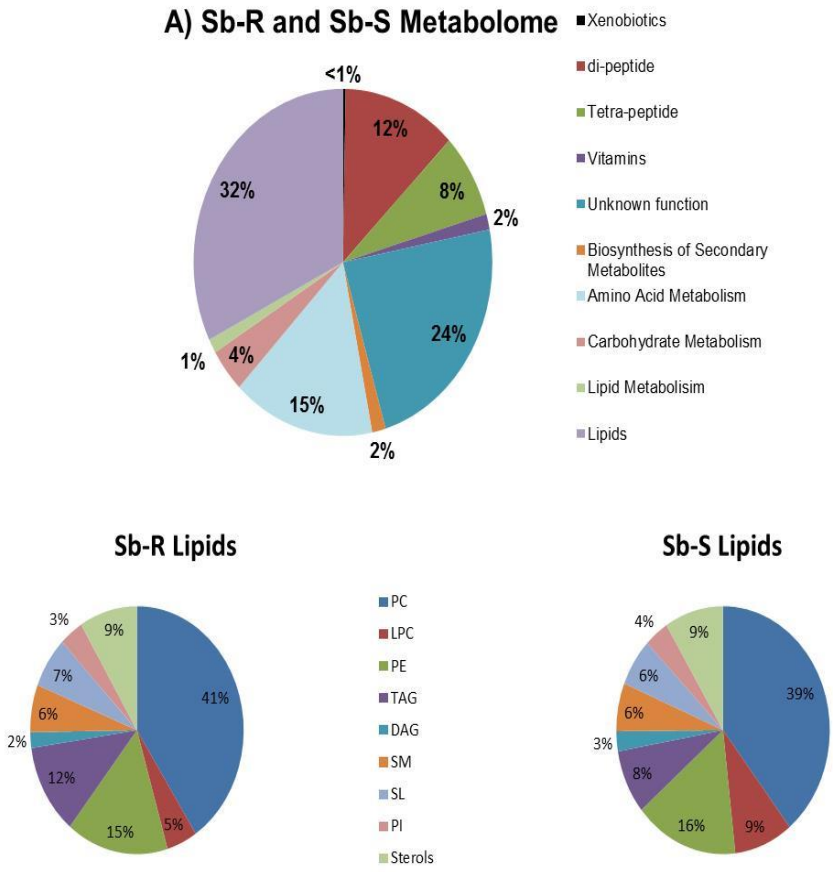


Figure 4.1 Pie charts depicting A) percentage of different classes of compounds that make up the metabolome of Sb-R and Sb-S WT *L. donovani* promastigotes. Metabolites were separated using a p-hilic column and identified using IDEOM software. Lipidome of Sb-R WT (B) and Sb-S WT (C) *L. donovani* promastigotes separated using a silica gel column and identified manually.

Twenty four percent of the metabolites annotated did not have a designated class or known function. The majority (67%) of the metabolome was classified as peptides, amino acid metabolites or lipids (Figure 4.1A). Classes such as vitamins, xenobiotics and metabolites involved in carbohydrate or secondary metabolite synthesis made up the remainder of the metabolome. The WT Nepalese strains used in this investigation had a lipid content of 32%. The exact percentage of lipids identified can vary between studies as a consequence of the extraction method and LC column used in the investigation. Using a hilic column has advantages in that it allows the separation of a wide variety of metabolites over the elution time of samples through the column. A major drawback however is that the separation of lipid species is poor. The entire lipid content of the cell is typically eluted in a two minute window at the very beginning of a twenty minute elution through a p-hilic column (Figure 4.2). Given that lipids account for 30% of the entire metabolome, the resolution of individual peaks corresponding to lipids is poor using this method. In addition, classification and identification of individual lipids by IDEOM can be inconsistent. This is in part to do with the poor resolution and overlapping of peaks but also partly due to the presence of multiple, lipid species that only differ by a double bond and two hydrogen atoms. For this reason, lipid analysis was performed using a silica gel column and annotated manually. These data sets were extracted at the same time as the metabolomic data sets using 1:1 chloroform: methanol mixture. The lipid content of cells was termed the “lipidome”.

The two WT strains had several consistent, significant differences in their metabolomic profiles. From over 1000 metabolites annotated using IDEOM, 22

were consistently and significantly up or down regulated between the Sb-S and Sb-R WT strains (p value < 0.05, Table 4.1). The majority (17) of the metabolites altered were either directly involved in amino acid metabolism, were amino acids or a dipeptide (two amino acids joined by a peptide bond). All but one of these metabolites was upregulated in the Sb-R WT strain compared to Sb-S WT. Only phenyllactic acid was significantly downregulated in the Sb-R WT strain compared to Sb-S WT. Of the remaining five metabolites, hypoxanthine and xanthine were downregulated in Sb-R WT while 4-hydroxysphinganine and 8-amino-7-oxo-nonanoic acid were upregulated in Sb-R WT compared to Sb-S WT. N-methylethanolamine-phosphate was consistently found in the Sb-R strain but always absent from the Sb-S WT parasite.

Table 4.1. Relative presence of significantly different metabolites in Sb-R/Sb-S promastigotes. Mean ratios shown are from three separate experiments with an n = 4 replicates per experiment. All metabolites listed were significantly different ($p < 0.05$) in all three separate experiments, Sb-R WT vs Sb-S WT values. Table adapted from similar data presented in (Shaw *et al.*, 2016) supplementary data.

Metabolite	**Ratio Sb-R/ Sb-S ± SD**	**Metabolite**	**Ratio Sb-R/ Sb-S ± SD**
Amino Acid Metabolism		**Dipeptides**	
Aspartic acid	3.3 ± 2.0	Asn-Asp	4.7 ± 0.0
Glutamic acid	3.7 ± 0.6	Asp-Asp	8.1 ± 1.4
Histidine	4.0 ± 0.0	Asp-Ser	4.0 ± 0.2
Homocysteine	4.2 ± 2.7	Glu-Gly	3.6 ± 0.6
Hydantoin-5-propionic acid	7.4 ± 3.0	Glu-Val	9.3 ± 1.0
Isoleucine, leucine	2.3 ± 1.0	Lys-Asp	3.6 ± 0.5
Phenyllactic acid	0.2 ± 0.2	Met-Asp	4.7 ± 0.9
Proline	5.0 ± 0.3	Phe-Asp	4.3 ± 0.5
Tryptophan	3.2 ± 0.0		
		Sphingoid bases	
Lipid Metabolism		4-hydroxysphinganine	5.1 ± 3.1
N-methyl-ethanolamine-phosphate	NF in Sb-S		
		Nucleotide Metabolism	
Vitamins and cofactors		Hypoxanthine	0.1 ± 0.0
8-amino-7-oxo-nonanoic acid	5.4 ± 0.0	Xanthine	0.5 ± 0.3

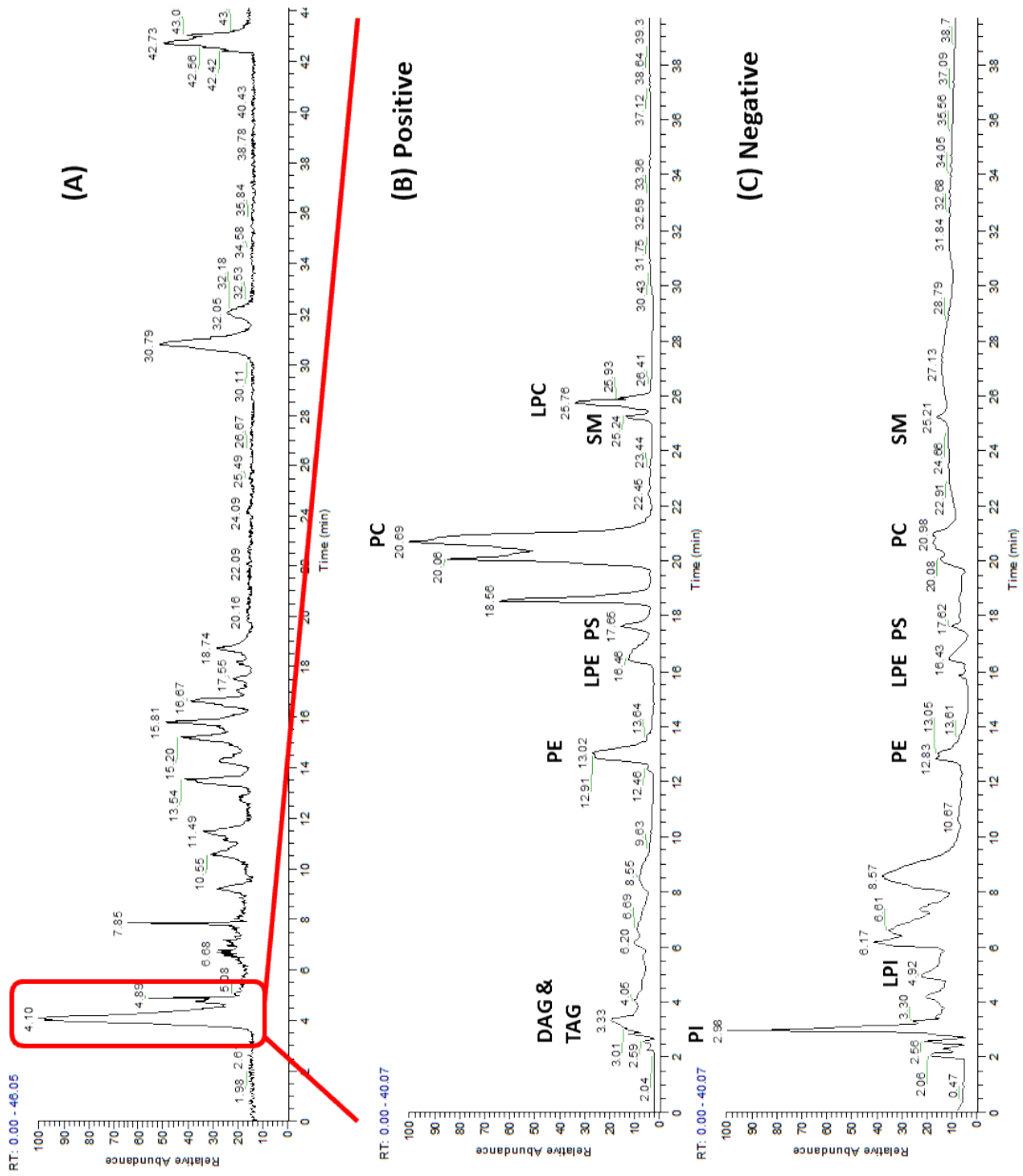


Figure 4.2. Chromatograms of *L. donovani* Sb-R WT promastigotes extracted using the appropriate solvents. A) Entire metabolome extracted using chloroform:methanol:water solvent and separated using a p-hilic column. The lipid content of the metabolome is represented inside the red rectangle. B) Positive and C) Negative lipidomic sample extracted using chloroform:methanol solvent and eluted through a silica gel column.

4.2.2 Lipid Differences Associated With Antimonial Resistance

The total number of lipid species consistently identified in each WT strain was comparable (194 in Sb-R WT and 191 in the Sb-S WT). Glycerophospholipids (PC, PE, LPC, TAG and DAG) make up 75% of the lipidome of our two *L. donovani* strains. The most abundant of these were PCs which accounted for 39-41% of the total phospholipid content. Sphingolipids (SL, SM, PI), where the backbone is a sphingoid base instead of a glycerol, accounted for 16% of the lipidome, while sterols accounted for the remaining 9% (Figure 4.1.B).

In relation to the numbers of each lipid species found, the major difference between the two lipidomes was observed in LPCs and TAGs. Seven TAGs were unique to the Sb-R WT strain. TAGs common to both strains were present in similar amounts in the two strains.

The number and relative intensity of LPCs, a derivative of PCs formed by phospholipase mediated hydrolysis of one of the fatty acid chains of a PC, differed significantly between the two WT parasites. Nine LPC species were consistently identified in both Sb-R WT and Sb-S isolates. An additional seven were unique to the Sb-S WT lipidome (Table 4.2). Those LPCs that were unique to Sb-S WT made up 33% of the total LPC content so their absence in Sb-R WT appears to be significant. There was no apparent correlation between the chain length or saturation state of the chains of the LPCs unique to Sb-S WT. The relative amount of only one common LPC differed between strains i.e. LPC (18:4), which was upregulated in in the Sb-R WT (relative ratio 2.6 ± 0.1, Table 4.2).

Table 4.2. Relative presence of PCs and LPCs in Sb-R WT and Sb-S WT promastigote parasites. Mean ratios shown are from three separate experiments with an n = 4 replicates per experiment, $p < 0.05$ for Sb-R WT/Sb-S WT. Table adapted from similar data presented in (Shaw *et al.*, 2016) supplementary data

LPC type	Mean Ratio WT Sb-R / WT Sb-S ± SD	PPC type	Mean Ratio WT Sb-R / WT Sb-S ± SD
LPC(18:4)	2.6 ± 0.1	PC(32:0)	0.3 ± 0.0
LPC(14:0)	Not detected in Sb-R	PC(33:0)	0.3 ± 0.1
LPC(18:1)	Not detected in Sb-R	PC(34:0)	0.2 ± 0.0
LPC(18:2)	Not detected in Sb-R	PC(34:1)	0.4 ± 0.0
LPC(19:0)	Not detected in Sb-R	PC(35:0)	0.2 ± 0.0
LPC(20:2)	Not detected in Sb-R	PC(35:1)	0.4 ± 0.0
LPC(22:4)	Not detected in Sb-R	PC(36:1)	0.3 ± 0.0
LPC(24:0)	Not detected in Sb-R	PC(37:1)	0.3 ± 0.0
		PC(38:2)	0.4 ± 0.0
		PC(38:3)	0.5 ± 0.0
		PC(34:5)	2.1 ± 0.0
		PC(35:5)	1.8 ± 0.5
		PC(35:6)	2.3 ± 0.6
		PC(36:6)	2.1 ± 0.6
		PC(36:7)	3.0 ± 0.3
		PC(39:8)	1.8 ± 0.4
		PC(40:9)	1.9 ± 0.5
		PC(40:10)	3.4 ± 0.07

PCs were the most abundant class of lipid identified in promastigotes for both WT parasites (Figure 4.1.B). The PCs identified had between 24 - 44 carbons in their side chains and different states of saturation based on number of carbon-carbon bonds present in the side chains, with twelve double bonds in the a PC being the maximum present. The total number of PCs identified in each strain was similar (79 in Sb-R WT and 75 in Sb-S WT). Sixty-nine of the PCs were common to both strains. Eleven PCs were unique to Sb-R WT and five were unique to the Sb-S WT (Table 4.3). There did not appear to be a particular pattern to the saturation state of PCs specific to either of the two strains. However, significant differences in the abundance of these common PCs were observed (Table 4.2). Eight PCs were significantly and consistently upregulated in Sb-R WT parasites compared to Sb-R WT (ratio > 1, Table 4.2), and ten were significantly down regulated in Sb-R WT compared to Sb-S WT (ratio < 1, Table 4.2). The PCs with higher amounts in the Sb-R WT strain were longer chained polyunsaturated lipids and all having between 34 and 40 carbons in their side chains, and between 5 and 10 carbon double bonds in their structure. In contrast, PCs found in significantly higher amounts in Sb-S WT were shorter chained i.e. 32-38 carbons and contained either none or very few carbon double bonds in their structure, i.e. a maximum of only 3 (Table 4.3) Three of the eight PCs were completely saturated [PC(32:0), PC(33:0), PC(34:0)], three had only one double bond [PC(34:1), PC(35:1), PC(36:1)], one PC had two double bonds [PC(38:2)] and one had a total of three double bonds [PC(38:3)]. Thus the overall trend was for Sb-R WT promastigotes to have a higher content of larger,

unsaturated PC whereas Sb-S WT parasites had a greater number of the smaller and more saturated PCs.

Table 4.3. Phosphatidylcholine (PC) species unique to either Sb-R WT or Sb-S WT *L. donovani* promastigotes when compared to the other.

Only found In Sb-R WT	Only found in Sb-S WT
PC(27:0)	PC(38:1)
PC(28:1)	PC(40:2)
PC(30:4)	PC(41:7)
PC(33:1)	PC(42:3)
PC(33:2)	PC(42:6)
PC(34:2)	
PC(39:7)	
PC(42:11)	
PC(44:10)	
PC(44:2)	
PC(44:3)	

4.2.3 Genomic Differences Associated With Antimonial Resistance

This study was part of the EU sponsored project, Kala-drug, which aimed to identify differences in clinical strains and relate them to genomic changes. I was responsible for culturing and isolation of DNA from WT parasites, and then sent the DNA to The Sanger Institute for sequencing. The genomic data was analysed by Dr Tim Downing and Dr Hideo Imamura.

As mentioned, the two WT strains were genetically very similar (Section 2.3 Parasites) therefore it is reasonable to postulate that any difference noted in the metabolism could be as a direct result of genetic differences (Downing *et al.*, 2011a). Several changes in chromosome copy number, single nucleotide

polymorphisms (SNPs), copy number variants (CNVs) and deletions were found between the two strains.

Seven differences in chromosome copy number of specific chromosomes were detected. Sb-R WT had higher doses of chromosomes 2, 8, 11 and 33 whereas the Sb-S WT strain had higher doses of chromosomes 6, 26 and 25. A total of 127 SNPs were found to differ between the two WT isolates. Thirty three of these were non-synonymous SNPs, 10 of which have previously been associated with Sb-R resistance in the ISC. Of the 18 local CNVs that differed between the two WTs from this study, 14 were in coding regions whereas 4 were found in non-coding regions. Sequencing also uncovered 38 deletions and 13 insertions differing between Sb-R WT and Sb-S WT.

Correlating all of the genomic changes to alterations of the metabolome is not possible as the function and identity of every gene in *L. donovani* has not been completed. There were several difference that were mapped to known genes that could explain metabolomic differences and possible differences in the susceptibility to Sb. Notably, a two base, out of frame, deletion in the aquaglyceroporin-1 (AQP1) gene in the Sb-R WT was found, suggesting that production of AQP1 in this strain would be absent. Another deletion was mapped on chromosome 31 (chr31) of the Sb-R isolate. This deletion encompassed several genes known to have a role in fatty acid and lipid metabolism, more specifically; a C-5 sterol desaturase (LdBPK_310620), triacylglycerol lipase (LdLip3-lipase, EC: 3.1.1.3, LdBPK_310860), and a lipase precursor (EC: 3.1.1.3, LdBPK_310870). This deletion would likely affect

a number of lipid enzymes and have a downstream effect on a number of metabolomic pathways.

4.3 Discussion

Initial experiments sought to map and compare the metabolome of Sb-S and Sb-R WT *L. donovani* promastigotes. Initial results indicated that a significant number of the differences occurred in phospholipids of the two strains. As lipids make up approximately 30% of the *L. donovani* metabolome and their resolution using a p-hilic column with standard metabolomic extraction solvents is poor, we sought to specifically extract lipids using a more lipophilic extraction and therefore separated the lipidome using a silica gel column. Both metabolomes and lipidomes were comparatively very similar to each with the proportions of metabolite and lipid classes only altering by a few percent. This is not surprising as sequencing of their genomes revealed that the two strains were genetically very similar (Downing *et al.*, 2011a). The proportions of metabolites and lipids that account for the metabolome and lipidome are also comparatively similar what has already been published (Zhang and Beverley, 2010). Given the genetic similarity between the two WT isolates, it is reasonable to postulate that any difference noted in the metabolism could be as a direct result of genetic differences. Correlating each nucleotide sequence change to a specific functional phenotypic change was difficult however as the function of every gene in the genome is not yet known. There was however several genomic differences that correlate with mechanisms that have previously been associated with Sb resistance; A deletion in the AQP1 gene sequence in the Sb-R WT would likely result in dysfunction of the gene and a loss of functional AQ protein. AQP1 is a member of the aqua porin super family and functions as a membrane channel transporter that allows transport of small solutes (Maharjan *et*

al., 2008). A number of previous studies have correlated a deletion in the AQP1 gene with reduced transport of Sb^{III} in to the parasite and therefore increased resistance to antimonials (Gourbal *et al.*, 2004, Marquis *et al.*, 2005, Maharjan *et al.*, 2008). Moreover, the deletion in chr 31 of the Sb-R WT, encompassed a number of lipid enzymes which may also describe a number of metabolomic differences also observed.

The most pronounced differences in lipids between Sb-R and Sb-S WT isolates were in the intensities and composition of LPCs, PCs and TAGs. LPCs are formed from phospholipase mediated hydrolysis of one of the fatty acid chains of a PC to form an LPC plus a fatty acid chain. Sb-S parasites were found to have 8 species of LPCs that weren't identified in Sb-R. The deletion in chr31 encompassed the gene for a lipase precursor. As cleavage of the fatty acid chain of a PC to form an LPC is lipase mediated, the deletion of the lipase precursor and subsequent reduction in lipase could directly impact the breakdown of PC to LPC in Sb-R WT. Along with the more numerous individual species of LPC identified in Sb-S, a decrease in the number of PC species was identified. Four PCs were identified in Sb-R that were not found in Sb-S. No LPCs were present in the culture media and no other mechanism for their synthesis is known. It is therefore likely that the disparities in LPCs and PC levels in the two isolates are linked and a reduction in a lipase cleaving a fatty acid would help to explain this disparity.

A significant difference in the DAG and TAG content of the two WT strains was also observed in this study. Sb-R WT had a significantly richer TAG content than the Sb-S

WT (12% of the lipidome compared to 8%). In contrast the DAG content of Sb-R was deficient compared to Sb-S (2% compared to 3%). This disparity may also be a consequence of the deletion on chr31 of Sb-R WT. Here, the gene encoding a triglycerol lipase, responsible for cleaving ester links at the start of the fatty acid tails in triglycerides, appeared to be lost in the deletion. The reduction in enzymatic activity upon the cleavage of TAGs could explain the high TAG content in Sb-R WT compared to that of Sb-S WT. TAG hydrolysis in Sb-R would be reduced with the deletion of TAG lipase and would also affect the DAG content as a reduction in the hydrolysis of TAGs to produce a DAG plus fatty acid chain would also occur.

Another important lipid change noted between promastigotes of Sb-R and Sb-S WTs was observed in their PC content. The Sb-R strain contained significantly greater intensities of long chain, polyunsaturated PCs. Conversely, the Sb-S WT line had significantly greater intensities of short chain, saturated PCs. Phospholipids are important components of cell membranes and its composition influences properties such as rigidity, permeability and space between lipid tails. PCs make up around 20% of the plasma membrane (Zhang and Beverley, 2010) and so an alteration in their content is likely to have a significant impact on plasma membrane characteristics. Lipids with shorter alkyl chains in their fatty acid tails make membranes more rigid and fluid whereas longer alkyl chains make more intermolecular interactions and fluidity is reduced. Saturation of the tail groups also influences the rigidity and fluidity of membranes. Unsaturated alkyl chains in the lipid bilayer increase the number of ‘kinks’ in the tails of phospholipids, disrupting the intermolecular interactions, compromising the rigidity of the membrane and

making it more fluid (Hsuchen and Feingold, 1973, Zhang and Beverley, 2010). Sb-R WT had more long chain polyunsaturated PCs compared to Sb-S WT. It is therefore difficult to postulate any difference in the fluidity of membranes between Sb-S WT and Sb-R WT. In theory, the long alkyl chains of Sb-R WT should make it less fluid than Sb-R WT but at the same time the polyunsaturation of the alkyl chains in Sb-R would make it more fluid than the Sb-S WT. *Leishmania* parasites resistant to several drugs including MIL, AmB or pentamidine have been shown to have a less fluid membranes and this change is associated with phospholipid composition (Rakotomanga et al., 2007, Jhingran et al., 2009, Mbongo et al., 1998a, Basselin et al., 2002). Changes in membrane composition can affect uptake of drug via plasma and mitochondrial membrane potential. Furthermore, a reduced fluidity in the plasma membrane has been associated with a resistance to oxidative stress from the host immune system. Sb has been shown to elicit oxidative stress as a mechanism of parasite killing and SSG resistant parasites have previously been shown to have enhanced resistance to oxidative stress compared to SSG susceptible parasites (Carter *et al.*, 2005).

In order to fully elucidate the impact of the mutation at chr31 on the levels of saturated PCs, LPCs and the number of TAG species, further analysis would be required. Quantitative PCR to check for the expression of genes (LdBPK_310620 and LdBPK_310860) after Sb drug pressure, or using medium that contains radiolabelled precursors of these lipids, could help elucidate their role in Sb resistance. Gene complementation/knock out could also be performed. Adding the genes the have

been deleted back in to the genome and reanalysing the metabolome to see their effect would help to validate the consequences of their deletion.

As well as a difference in lipids, the Sb-R WT had significantly greater amounts of metabolites involved in the metabolism of amino acids and a number of dipeptides. *Leishmania* are auxotrophic for several amino acids and therefore are required to obtain them from the environment. All of the amino acids found in increased levels in the Sb-R WT were present in the culture media used. Therefore it is likely that the observed difference in amino acid levels between the two strains is due to increased uptake from the media. Aspartic acid (Asp) was significantly upregulated in the Sb-R WT and its backbone was present in six of the eight dipeptides that were also significantly upregulated. Asp serves as a precursor to methionine, threonine, isoleucine, and lysine. So it is perhaps not a coincidence that isoleucine was also significantly upregulated in Sb-R WT. This increase in amino acids and dipeptides is difficult to correlate with specific metabolic pathways as their use is widespread. Indeed the increased acquisition could simply be a by-product of an increase membrane fluidity associated with increased drug efflux and thus increased drug resistance. Previous studies have also found correlations in an increased uptake of several unrelated metabolites, while not increasing drug uptake, in other drug resistant *Leishmania* (Basselin *et al.*, 1997). Proline was present in significantly higher levels in Sb-R parasites. This amino acid is known to be a scavenger of free radicals and can enhance cell survival in response to stress (Kaul *et al.*, 2008). In *Leishmania*, proline levels were shown to be increased in purine starved cells and

when subjected to osmotic stress, it was also required for volume recovery post stress (Inbar *et al.*, 2013, Martin *et al.*, 2014). Importantly, supplementing promastigotes of *L. donovani* with proline was shown to lend an increase in tolerance towards Sb^{III} (Berg *et al.*, 2015). Our Sb-S isolates are sensitive to both Sb^{VI} and Sb^{III} whereas Sb-R is resistant to both antimonials. Antimony resistance in *L. braziliensis* has been strongly correlated with an increase in resistance to oxidative stress (Andrade and Murta, 2014). Although it is known to scavenge free radicals, the exact mechanisms of the protective effects of proline are not yet fully understood. The published data, along with the notable increase in proline in our Sb-R isolates in this study add credence to the hypothesis that proline plays a role as a stress-response metabolite and in particular against the effects of oxidative stress.

N-methylethanolamine was not identified in Sb-S WT indicating that this parasite is inherently deficient in that isolate, which could imply that the choline and ethanolamine branches of the Kennedy pathway (see Figure 5.8 in Chapter 5) between phosphocholine and phosphoethanolamine do not exist. PC and PE synthesis in *L. donovani* are closely linked, with multiple points of cross reactivity between the two branches. Whether this is indicative or the Sb-S background or unique to this particular strain cannot be postulated as it is the only example of a Sb-S strain analysed in this study.

An important consideration in interpreting the results of this study and its clinical relevance is the fact that the promastigote stage was used. Promastigotes replicate

in the sandfly host, and it is only present at the beginning of the life cycle in the mammalian host, where the amastigote stage which lives within phagocytic cells predominates. Ideally, any work to assess the metabolome would be done on the amastigote stage but e*x vivo* amastigotes would be difficult to analyse because of various technical issues. Although molecular biochemical studies can be performed on axenic amastigotes, metabolomic profiling proves difficult. Firstly, one replicate for extraction of the metabolome requires 4 x 10^7 parasites. Therefore acquiring sufficient numbers of parasites for quantitative analysis may require an unacceptable number of mice or hamsters if the parasites are derived from hamsters or a significant amount of cell culture if *in vitro* derived amastigotes are used. Secondly, purification of intracellular amastigotes to remove any host macrophage contaminants may be problematic as it would be difficult to type individual compounds present as host or parasite derived. This would be further complicated if the metabolism of an uninfected and infected macrophage differed. This is likely to occur as expression of genes is already known to vary between uninfected and infected macrophages (Filardy *et al.*, 2014). In addition, a subtle increase in a metabolite for one cell (e.g. the parasite) may be masked by the presence of a large amount of this metabolite in the other cell (e.g. the macrophage). Rupturing the macrophage membrane before harvesting the amastigotes to remove any macrophage derived metabolites is perhaps a way around this. However, identifying a gentle method that does not damage the integrity of the parasite or macrophage would add extra steps and extend the time before the sample could be quenched and the whole metabolism arrested, and thus

such a procedure runs the risk that the results would not reflect the true metabolome of the cell. At present, the use of promastigotes appears to be the most appropriate stage and it allows the methods associated with analysis of the data to be refined. The results can be compared to genomic DNA, and as gene expression is modulated at the post-transcription level, therefore allowing some comparative analysis to be completed. A compromise would be to use axenic amastigotes but analysis of the transcriptome has already identified significant differences in the results for axenic and lesion derived amastigotes, indicating that axenic amastigotes may not give the same results as *in vivo* amastigotes (Holzer *et al.*, 2006).

A similar metabolomic approach was used in a previous study using the same WT strains and its initial findings were published by (t'Kindt *et al.*, 2010). Both t'Kindt's study and ours have similarities in their results but there are also some key differences. Firstly, a discrepancy in the number of metabolites identified as altering significantly between the strains of WT parasite exists. The study by t'Kindt *et al.* identified 100 differences in the levels of metabolites between the two isolates, whereas this study has only declared just under half of that. A number of reasons could be a factor in this discrepancy. Firstly, t'Kindt's data (2010) appears to have been generated from only the one experimental replicate (t'Kindt *et al.*, 2010). Although 4 biological replicates were performed in the experiment, an experimental replicate does not appear to be evident. In this study we repeated the same experiment with four biological experiments on three separate occasions. Metabolites were only considered if they were identified in all three replicates and

were only considered to be altered if the change was significantly found in all three experiments. Although this selection threshold is high, it weeds out metabolite changes that are inconsistent. Several metabolites, including those involved in the oxidative responses are highly volatile and can alter significantly between experiments. Additionally, the variability in t'Kindt's experiment seems high. Of the 300 metabolites identified, 100 were found to significantly differ between the Sb-S and Sb-R strains. This seems surprisingly high when the genetic backgrounds of the two strains are apparently so close. Normalising the metabolite intensities to several housekeeping genes could be a factor in this high variability. Moreover, t'Kindt included lipids in their metabolome comparison whereas this study analysed the lipidomes of the two strains separately from the metabolome. As shown in Figure 4.1 and 4.2, lipids account for one third of all metabolites yet is eluted in a small, two minute window at the start of metabolome separations using a p-hilic column. The result of this is poor separation, resolution and identification of the different lipids in the metabolome. It is therefore highly beneficial to extract and separate the lipidome independently, using a more suitable column. By analysing the lipidome separately we identified over 140 different lipid species compared to the 55 reported by t'Kindt. Accurate identification of the saturation of the lipids identified is also more robust. This therefore gives this study much greater scope for analysing different lipid classes and the subtle differences in saturation of the sidechains of the lipids identified.

This study has demonstrated how metabolomic profiling of closely related isolates can tease out subtle differences in the intensity and composition of metabolites

found in clinical isolates of *L. donovani*. Moreover, several of the observed metabolomic differences correlate with differences observed in the genome of the two strains. It also provides a baseline of the metabolomic profile of the two isolates to allow the study of acquired resistance to other drugs such as MIL or PMM.

Absolute identification of metabolites, without using fragmentation is not possible. Even when standards are run, certain metabolites with the same chemical formula can overlap retention times. Despite this, we can be suitably confident in the identification of metabolites identified to differ in this experiment. Several of the metabolites identified to differ between strains were included in the standard mix used. Moreover, there is a certain amount of overlap and corroboration between metabolites, for example, patterns in alterations of lipid metabolism metabolites. The identity of lipids is more certain than that of our metabolites. Extraction using lipid specific solvents and separation using a silica gel column removes 75% of the metabolome therefore reducing interference, signal disruption and ion suppression from other non-lipid metabolites. It also increases the signal resolution for the 25% of the metabolome that is left. Moreover, the retention times for each class of lipid can be more accurately predicted so the chance of two different classes of lipids, sharing a chemical formula, overlapping is significantly reduced. This allows us to be confident of the class of lipid and the exact formula which makes predicting the lipid accurate.

In summary, the main objectives and results achieved in this chapter were

- WT isolates with different background susceptibilities to Sb were analysed for differences in their metabolomes, lipidomes and genomes.
- The most pronounced differences between strains was found in the composition of lipids, particularly PCs, LPCs, DAGs and TAGs
- Sb-S WT was found to contain higher intensities of short chain, saturated PCs compared to Sb-R WT
- Several differences in the composition of lipids correlate with observed differences in the genome, including a deletion in chr31 in Sb-S
- Metabolomic and lipidomic analysis using LC-MS can provide an in-depth analysis of the metabolite and lipid content of *L. donovani* promastigotes that can be correlated with genomic studies.

Chapter 5. The Effect of Miltefosine Selection on the Metabolome and Lipidome of *L. donovani*

5.1 Introduction

Miltefosine was first introduced as an alternative to antimonial treatment of VL in the ISC in 2003 after demonstrating effective cure in 95% of cases as part of a randomised, open label comparison with AmB (Sundar et al., 2002b). A subsequent study showed MIL was effective in the treatment of children suffering from VL (Singh *et al.*, 2006b). Oral administration of MIL makes it ideal for use in the ISC as patients can self-administer. This decreases the overall cost of treatment mainly because trained personnel are not required to administer the drug parentally. There are however, disadvantages to using MIL; A long treatment regimen (28 days) is required and its long half-life means that sub therapeutic levels of the drug remain in the patient for a number of weeks post treatment. This can help craft conditions that allow parasites to develop tolerance or resistance to MIL. Whether or not compliance of treatment is a particular issue at present is perhaps a matter of debate. A 2013 study encompassing 171 patients in Nepal reported that only 83% of patients adhered to the correct treatment protocol (Uranw *et al.*, 2013). Conversely, another Nepalese study found compliance to be greater than 95%, suggesting that non-compliance of MIL treatment is not an issue (Rijal et al., 2013). Despite this ambiguity regarding compliance, the efficacy of MIL in the ISC does appear to be waning in both India and Nepal (Sundar *et al.*, 2012, Rijal *et al.*, 2013). With the lack of other options currently available or in the pipeline for VL treatment, extending the clinical life of MIL is of major importance.

Perhaps encouragingly, MIL resistant isolates have not yet been identified in clinical strains however MIL resistance can be routinely selected for in laboratory

maintained parasites. Thus far, laboratory selected MIL resistance has always been associated with decreased drug accumulation within the parasite, via one of two mechanisms. The first involves the LdMT transporter and its LdRos beta subunit. Deletions or mutations of the genes encoding either of these proteins causes the complementary protein to become translocated, causing a reduction in MIL uptake and subsequent MIL tolerance (Perez-Victoria *et al.*, 2003b, Perez-Victoria *et al.*, 2006). The importance of the LdMT gene in other species of *Leishmania* has also been demonstrated in drug resistant *L. major* (Coelho *et al.*, 2012a). Converse to the reduced uptake caused by LdMT alterations, an increased efflux of the drug in drug resistant parasites has been correlated with the ABC family of transporters. This family includes several transporters that have previously been associated with drug resistance such as the MDR1 transporter and P-glycoprotein-like transporters (Perez-Victoria *et al.*, 2001, Seifert *et al.*, 2003). MIL is known to disrupt lipid and fatty acid metabolism in MIL sensitive cells, specifically membrane PC and PE (Rakotomanga *et al.*, 2007, Rakotomanga *et al.*, 2005). It is therefore not surprising that resistant parasites have shown to have reorganised their lipid content when made resistant to MIL in the laboratory. So far, to date, the only metabolomic study focusing on MIL resistance uncovered an increase in the level of several amino acids, and it was suggested that this promoted the survival of drug resistant parasites in its macrophage host (Canuto *et al.*, 2012).

As with the antimony sensitive and resistant isolates in the previous chapter, we used a metabolomic approach to map the metabolome of parasites made resistant to MIL. Understanding the metabolites and biochemical pathways altered in MIL-R

parasites compared to WT may help identify strategies, such as using drugs synergistically with MIL, or allow co-treatment with inhibitors which block pathways that allow MIL resistance (e.g. efflux pump inhibitors). In tandem with genomic sequencing, we sought to elucidate possible mechanisms of resistance by linking alterations in the genome with those in the metabolome of MIL-R parasites. Moreover, we sought to investigate if it was possible to identify metabolic markers that could be used to type MIL-R parasites. Such a strategy would allow improved identification of MIL-R parasites and help select the most appropriate course of chemotherapy. This would not only improve patient outcome but could potentially extend the clinical life of MIL.

Therefore, the main objectives of this study were:

1) Analyse metabolome and lipidome alterations associated with selection of MIL resistance in Sb-S and Sb-R parasites.

2) Link any metabolomic alterations with observed changes in the genome of MIL-R parasites.

3) Determine if MIL resistance is associated with lower drug levels within selected MIL-R parasites.

5.2 Results

5.2.1 Metabolomic changes associated with MIL resistance

To identify changes in metabolites associated with the selection of MIL resistance, the metabolome (excluding lipids) of MIL-R parasites was compared to that of their WT parent. A total of 27, non-lipid metabolites consistently differed between at least one of Sb-S MIL-R or Sb-R MIL-R compared to their WT parent ($p < 0.05$). Twenty metabolites were found to be significantly upregulated in the Sb-S MIL-R line compared to Sb-S WT ($p < 0.05$, Table 5.1). A PCA analysis based on the entire identified metabolite content visualised the major differences between samples (Figure 5.1). One biological replicate for Sb-S WT was removed due to signal intensity drift but all other biological replicates for the four strains appeared clustered with other replicates of the same strain, a sign of low variance between biological replicates. The clusters of Sb-R WT (green dots) and Sb-R MIL-R (red dots) were markedly closer to each other compared to Sb-S WT (turquoise dots) and Sb-S MIL-R (blue dots) clusters. This is reflected in the lower number of metabolomic changes observed between Sb-R WT and MIL-R compared to Sb-S WT and MIL-R.

The majority of metabolites that differed were either amino acids or metabolites involved in lipid metabolism which accounted for 20 out of the 29 changes. No metabolites were consistently downregulated in Sb-S MIL-R parasite. Ten metabolites were common to both MIL-R strains; imidazol-5-yl-pyruvate, xanthine, choline phosphate, stearoylglycerone phosphate, phosphodimethylethanolamine, methionine, serine, 4-Nitrophenyl-3-ketovalidamine and phenolphthalein, were all

upregulated in both MIL-R clones. Two metabolites were significantly altered in both MIL-R clones but in contrasting amounts. Glycero-3-phosphocholine and (R)-AMAA were upregulated in Sb-S MIL-R but downregulated in Sb-R MIL-R. Ethanolamine phosphate was significantly downregulated in Sb-R MIL-R but was not significantly altered in Sb-S MIL-R. N-methylethanolaminephosphate was found significantly downregulated in Sb-R MIL-R parasites but was never identified in Sb-S isolates of *L. donovani*.

Nine of the 27 metabolites significantly altered in all experiments were involved in lipid metabolism. When these metabolites were mapped using the KEGG pathway in *L. donovani* the majority were localised to the Kennedy pathway (Figure 5.5). This pathway is responsible for the metabolism of both PE and PC in eukaryotes. In Sb-S MIL-R, there was a clear pattern that all metabolites of the Kennedy pathway were upregulated when MIL resistance was acquired, specifically the three metabolites in the choline branch of the Kennedy pathway i.e. choline, choline phosphate and CDP choline were significantly upregulated ($p<0.05$, Figure 5.2). Metabolites in this arm of the Kennedy pathway could be upregulated by an increased production of metabolites that feed into this pathway. There was evidence that this was the case for Sb-S MIL-R as glycerol-3-phosphate, which feeds in to the choline branch, was significantly upregulated compared to Sb-S WT. In contrast to this, in Sb-R MIL-R, there was evidence that the ethanolamine branch of the Kennedy pathway was downregulated in parallel with the choline branch being upregulated. Phosphodimethylethanolamine, part of the link between the choline and ethanolamine branches of the pathway, was also significantly upregulated in Sb-R

MIL-R compared to Sb-R WT (Figure 5.10). In Sb-S parasites the ethanolamine branch of the Kennedy pathway would appear not to be linked to the choline branch as N-methylethanolamine was consistently absent in the Sb-S parasites whereas its presence in Sb-R suggests that this link between the two branches remains intact in these parasites.

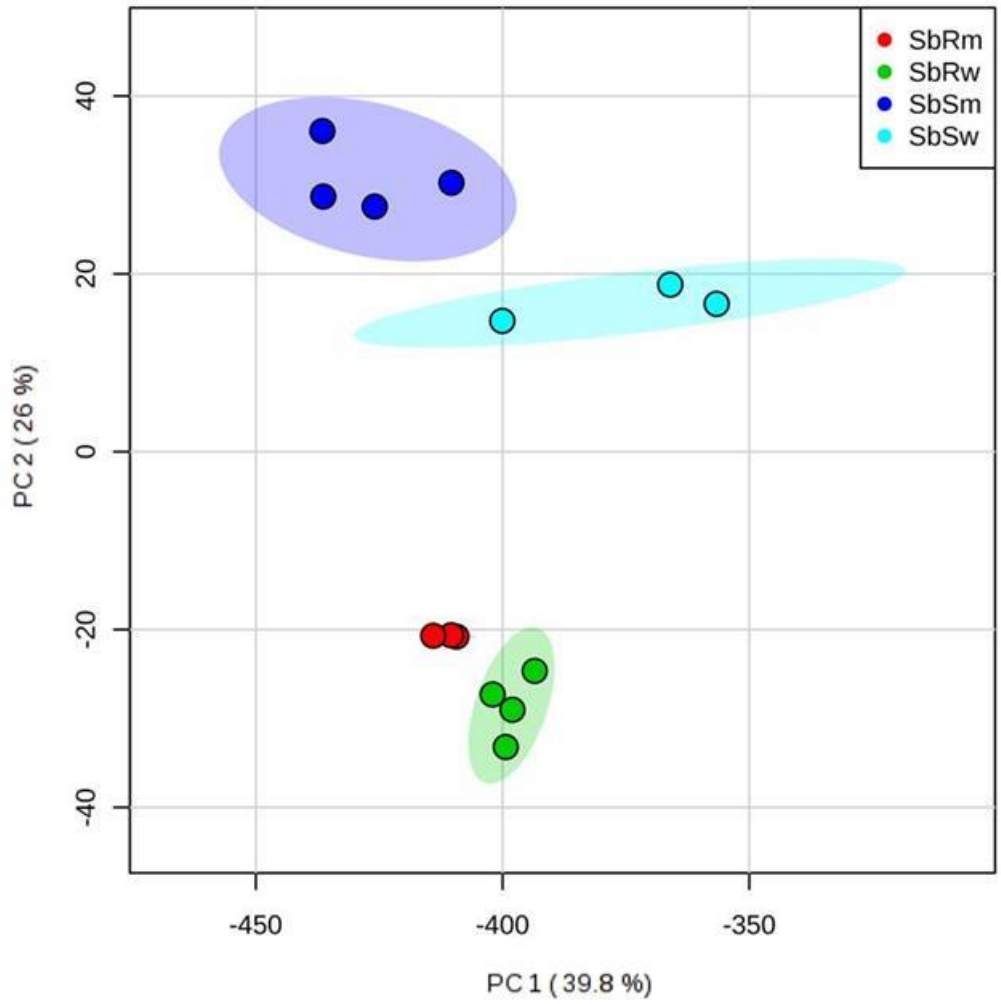


Figure 5.1. Principal component analysis distinguishing Sb-S WT (turquoise dots), Sb-S MIL-R (blue dots), Sb-R WT (green dots) and Sb-R MIL-R (red dots). Analysis was based on the peak intensity concentrations generated from IDEOM with one replicate for Sb-S WT removed due to signal intensity drift. Figure generated using MetaboAnalyst 3.0 (Xia *et al.*, 2015).

Table 5.1. Metabolites differentially regulated ($p<0.05$) in WT and MIL-R promastigotes. A value of >1 shows an upregulation and a value < 1 downregulation in MIL-R lines. Table adapted from similar data presented in (Shaw et al., 2016) supplementary data.

Metabolite	Mean Ratio Sb-S MIL-R/ WT ± SD	Mean Ratio Sb-R MIL-R/ WT ± SD
Amino Acids		
4-Nitrophenyl-3-ketovalidamine	2.1 ± 0.3	1.8 ± 0.3
D-Phenylalanine	2.0 ±.0.2	
GammaGlutamyl Glutamicacid	2.1 ± 0.5	
Imidazol-5-yl-Pyruvate	2.5 ± 0.7	1.9 ± 0.1
Methionine	1.9 ± 0.3	2.7 ± 0.1
Serine		3.1 ± 0.6
Tyrosine	2.6 ± 0.8	
Leu-Val	2.3 ± 0.2	
Leucyl-leucine	2.4 ±0.3	
Met-Ala	3.0 ± 1.3	
Nucleotide Metabolism		
Hypoxanthine	4.6 ± 1.6	
Uridine	2.9 ± 0.6	
Xanthine	4.3 ± 0.3	1.7 ± 0.1
Biosynthesis of Secondary Metabolites		
Taxadienalphayl acetate	3.6 ± 0.0	
Phenolphthalin	2.3 ± 0.2	1.3 ± 1.3
(R)-AMAA	2.0 ± 0.0	0.6 ± 0.1
Carbohydrate Metabolism		
Ala-Met-Trp-Asp	2.5 ± 0.4	
Lipid Metabolism		
Choline	2.0 ± 0.7	
Choline phosphate	1.9 ± 0.3	1.7 ± 0.2
Ethanolamine phosphate		0.2 ± 0.0
N-methylethanolamine phosphate	Not found	8.6 ± 0.2
Phosphodimethyl ethanolamine	3.2 ± 1.3	2.0 ± 0.5
Glycero-3-phosphate	2.7 ± 0.7	
Glycero-3-Phospho-1-inositol	2.1 ± 0.2	
Glycero-3-phosphocholine	1.8 ± 0.3	0.7 ± 0.3
Stearoylglycerone phosphate	3.6 ± 0.8	1.9 ± 0.1

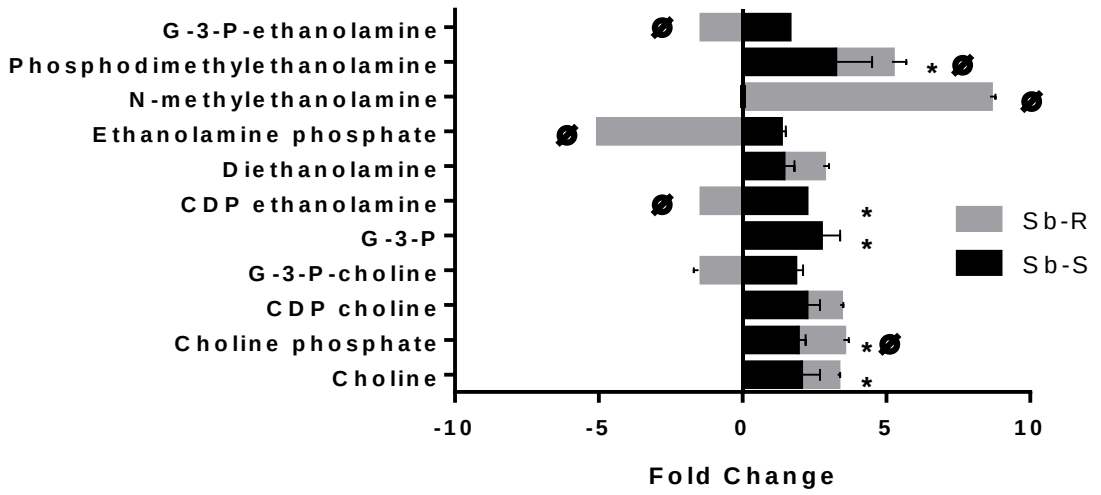


Figure 5.2. Alteration in metabolites involved in the Kennedy pathway in MIL resistance compared to WT counterparts for Sb-S and Sb-R *L. donovani* parasites. A fold change as a positive number represents an increase in metabolite level in the MIL-R compared to WT strain. A fold change as a negative number indicated a decrease in metabolite level in the MIL-R compared to WT strain. *$p < 0.05$ between MIL-R and WT in the Sb-S strain. Ø $p < 0.05$ between MIL-R and WT in the Sb-R strain. P values calculated using a student's independent t-test for multiple testing using the Benjamini-Hochberg method. G-3-P: Glycerol-3-phosphate, G-3-P-ethanolamine: Glycero-3-phosphoehtanolamine, G-3-P-choline: Glycero-3-phosphocholine.

5.2.2 Lipidomic Changes Associated With Miltefosine Resistance

Both MIL-R clones displayed significant alterations to metabolites involved in lipid metabolism, specifically metabolites involved in the biosynthesis of PCs and PEs. As before, in order to increase the resolution, identification and quantitation of lipids, the lipidome of parasites was extracted separately, passed through a silica gel column and analysed independently of the metabolome. Significant changes associated with MIL resistance were observed in PCs, LPCs, PEs and SLs in at least one of the MIL-R strains compared to its WT. Of these groups, PCs were the most affected group of lipids.

In general, the profile of PCs identified in MIL-R strains compared to their WT was similar. Saturated and unsatured PCs ranging from 26 - 44 total carbons in the fatty acid sidechains were identified. The lipid species identified in WT and MIL-R parasites of the same strain were identicatal but differed in their intenities of PCs. As a general trend, almost all PCs were found in significantly higher intensities in the Sb-R MIL-R compared to the WT. Several, significant alterations to PC and LPC intensities were found in both Sb-S and Sb-R MIL-R clones ($p < 0.05$, Tables 5.2 and 5.3). Eleven PCs were signifcantly upregulated in Sb-S MIL-R while 10 were significantly downregulated in Sb-R MIL-R (Table 5.2). There was no common PC that was found to be significantly up or down regulated in both of the MIL-R lines compared to their WT. All of the PCs found to differ between Sb-S MIL-R and WT were downregulated and more specifically were long chain PCs and highly unsaturated (at least 39 carbons in the sidechains and 4 to 10 carbon double bonds). In contrast, all significantly different PCs in Sb-R MIL-R were upregulated

and were either saturated or had only a small number of carbon to carbon double bonds (maximum of 4).

Table 5.2. Differences in the relative intensity of PCs found in promastigote parasites of Sb-R WT and its Sb-R MIL-R, and Sb-S WT and Sb-R MIL-R ($p < 0.05$). Mean ratios shown are from three separate experiments and 4 replicates/treatment were used in each experiment. A ratio > 1 corresponds to an upregulation in the MIL-R clone and < 1 corresponds to a downregulation in the MIL-R clone vs WT. In all cases $p < 0.05$ WT vs MIL-R clone. Table adapted from similar data presented in (Shaw et al., 2016) supplementary data.

Sb-R MIL/WT		**Sb-S MIL/WT**	
Chain length	**Mean Ratio**	**Chain length**	**Mean Ratio**
PC(39:5)	0.4 ± 0.2	PC(32:0)	2.6 ± 1.5
PC(39:6)	0.5 ± 0.2	PC(32:1)	1.6 ± 0.3
PC(39:7)	0.4 ± 0.1	PC(33:0)	2.6 ± 1.4
PC(40:4)	0.5 ± 0.2	PC(35:0)	4.1 ± 2.8
PC(40:5)	0.4 ± 0.1	PC(35:2)	1.3 ± 0.2
PC(40:10)	0.5 ± 0.3	PC(36:2)	1.7 ± 0.2
PC(42:7)	0.4 ± 0.2	PC(36:3)	1.5 ± 0.1
PC(42:8)	0.4 ± 0.1	PC(40:2)	2.3 ± 0.6
PC(42:9)	0.4 ± 0.2	PC(40:3)	1.7 ± 0.3
PC(44:12)	0.2 ± 0.2	PC(42:3)	2.1 ± 0.7
		PC(44:4)	2.8 ± 0.8

LPCs, normally formed when one of the fatty acid chains of a PC is hydrolysed, were also found to be significantly upregulated in response to selection of MIL resistance (Table 5.3).. Four LPCs were significantly upregulated in Sb-S MIL-R compared to its WT. In Sb-R MIL-R, two of these LPCs [LPC (16:0) and LPC (18:0)] were also significantly upregulated compared to WT. It is perhaps pertinant to note that LPC

(16:0) is structurally very similar to MIL. An additional lysophosphatidylcholine, LPC(17:0) was also upregulated in Sb-R MIL-R compared to WT with a further 2 LPCs significantly downregulated in MIL-R compared to WT.

PEs, DAGs, TAGs, SMs, SLs and other minor lipid classes such as prenols were analysed for alterations occuring as a result of selection of MIL resistance. Of these, only PEs, SLs and SMs of Sb-R MIL-R were found to differ significantly compared to Sb-R WT while no alterations in the intensities between Sb-S MIL-R and WT were found. Sphingolipids and sphingomyelins are important classes of compounds that are used in cell signalling as well as a major components of cell membranes. They also provide a source of ethanolamine phospate in the Kennedy pathway in *Leishmania*. Of the SLs or sphingoid bases identified in WT and MIL-R Sb-R, 15 were found to be consistently and significantly downregulated (Table 5.4). Of these, eight were different species of SMs and five were ceramides.

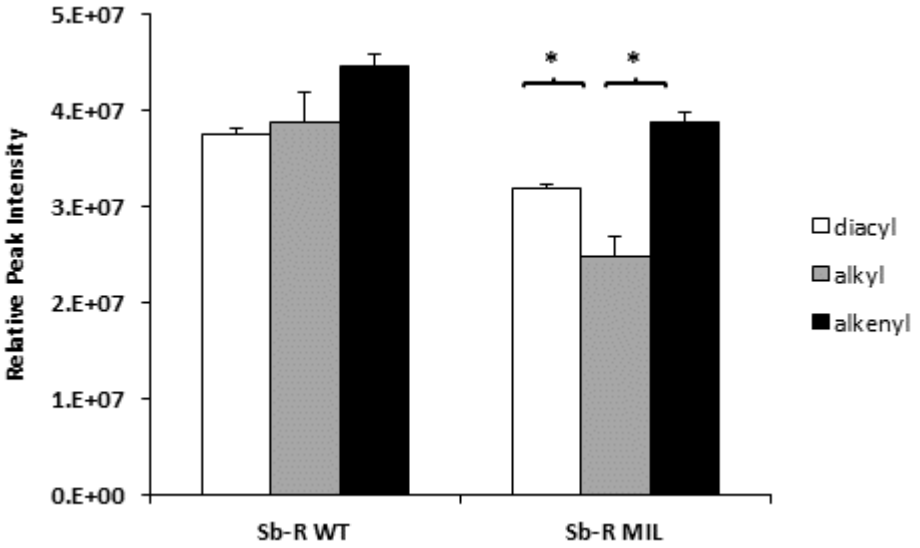


Figure 5.3. The relative intensity of the lipid anchors present in PEs found in Sb-R WT and Sb-R MIL-R parasites. PEs were identified after eluing lipidomic extracts through a sillica gel column LC-MS. Individual PEs were annotated mannually and grouped based on the linkages between their lipid anchors. * $p < 0.05$.

No individual PE species was significantly up or downregulated in either MIL-R parasite and the total PE content of MIL-R parasites and their respective WT counterpart was comparable. There was however a significant difference in the total PE content of Sb-R MIL-R compared to Sb-R WT when PEs were analysed on the basis of their lipid anchor (Figure 5.3). Post MIL induction, the total PE pool of Sb-R MIL-R parasites was observed to have a noticable decrease in the proportion of 1-alkyl-2-acyl-PEs coupled with a marked increase in diacyl- and 1-alkenyl-2-diacyl-PEs. This is indicative that while the quantity of PEs produced in Sb-R MIL-R was similar to Sb-R WT, the way in which they were synthesised had altered.

Table 5.3. Comparisons of LPCs found in WT and their corresponding MIL-R clone for *L. donovani* Sb-S and Sb-R promastigotes parasites. Mean ratios shown are from three separate experiments and 4 replicates/treatment were used in each experiment. A ratio > 1 corresponds to an upregulation in the MIL-R clone and < 1 corresponds to a downregulation in the MIL-R vs the relevant WT. In all cases, $p < 0.05$ corresponds to the WT vs the relevant MIL-R comparison. Table adapted from similar data presented in (Shaw et al., 2016) supplementary data.

Sb-R MIL/WT		**Sb-S MIL/WT**	
Chain length	**Mean Ratio ± SD**	**Chain length**	**Mean Ratio ± SD**
LPC(16:0)	2.4 ± 1.4	LPC(16:0)	7.4 ± 3.9
LPC(17:0)	2.4 ± 1.0	LPC(18:0)	7.5 ± 3.2
LPC(18:0)	3.1 ± 1.4	LPC(19:0)	10.5 ± 4.7
LPC(22:5)	0.4 ± 0.2	LPC(26:0)	4.0 ± 1.8
LPC(22:6)	0.5 ± 0.4		

Table 5.4 Alterations in the intensities of sphingolipids of Sb-R MIL-R compared to WT. Mean ratios shown are from three separate experiments and 4 replicates/treatment were used in each experiment. A ratio > 1 corresponds to an upregulation in the MIL-R clone and < 1 corresponds to a downregulation in the MIL-R vs the relevant WT. In all cases, $p < 0.05$ corresponds to the WT vs the relevant MIL-R comparison. CM = ceramide, SM = sphingomyelin.

Sphingolipid	Mean ± SD
CM (34:1) N-(hexadecanoyl)-sphingenine	0.6 ± 0.1
CM (34:0) N-(hexadecanoyl)-sphinganine	0.4 ± 0.0
CM (36:0) N-(octadecanoyl)-sphinganine	0.4 ± 0.0
Hydroxysphingenine	0.5 ± 0.0
Sphingadienine	0.5 ± 0.4
Sphinganine	0.5 ± 0.0
Sphingosine	0.6 ± 0.2
SM(32:1)	0.5 ± 0.0
SM(34:2)	0.5 ± 0.1
SM(36:1)	0.5 ± 0.1
SM(38:1)	0.5 ± 0.0
SM(40:1)	0.5 ± 0.0
SM(40:2)	0.5 ± 0.0
SM(41:2)	0.5 ± 0.0
SM(42:1)	0.5 ± 0.0

5.2.3 The Effect of MIL resistance on MIL uptake

Resistance to MIL in the laboratory has always been associated with a decreased accumulation of the drug inside resistant parasites. To assess if decreased accumulation was also observed in our MIL-R lines, MIL levels were determined in WT and MIL-R promastigotes using LC-MS. The peak corresponding to MIL had a retention time of approximately 26.5 minutes with a mass to charge ratio of 408.32 m/z.

In initial experiments, MIL levels in WT and MIL-R clones was determined 20 minutes post MIL treatment to determine if there was a difference in drug uptake between WT and MIL-R parasites (Figure 5.4). Both Sb-S and Sb-R MIL-R parasites had significantly lower peak intensities of MIL inside the cell compared to WT parasites ($p < 0.001$). Indeed, based on the size of the peaks, MIL-R parasites appeared to contain only a negligible amount of MIL but this may have been a consequence of a number of factors ranging from the short drug exposure time, rapid efflux or a reduced uptake of the drug. Due to the qualitative nature of metabolomics, a true quantitative comparison between MIL levels in parasites could not be achieved without running a calibration curve specifically for MIL in parallel with uptake experiments. Figure 5.5A shows the relationship between MIL concentration and peak intensity (AUC) at each time point. A linear correlation between AUC and MIL concentration was obtained for the MIL standards. This standard curve was used to calculate the exact MIL concentration within experimental samples. For all experiments, calibration curves were only used if the R^2 value of the line of best fit was >0.97. Promastigotes were incubated with 7 µM

MIL for 120 minutes and lipid extracts taken at time 0, 30, 60 and 120 minutes. A final time point of 120 minutes post MIL treatment was chosen because after this time Sb-S WT parasites began to show signs of cells stress and cell death. Data from 3 separate experiments showed that both MIL-R parasites had significantly lower amount of intracellular MIL compared to the relevant WT at each time point ($p < 0.05$, Figure. 5.6).

Both Sb-S and Sb-R MIL-R clones had significantly less MIL inside the cell at all three time points (Figure 4.6 B and C). By time 120 minutes, the difference in internalised MIL was almost 9 times greater than that inside Sb-R WT compared to MIL-R (7.39 vs 0.85 µg/ml MIL) and over 40 times greater inside Sb-S WT compared to MIL-R (6.39 vs 0.15 µg/ml MIL). Interestingly, the quantity of MIL inside WT parasites exceeded the concentration of the MIL present in the medium (7 µM), suggesting that WT parasites were actively uptaking MIL from the media. Data indicated that both WT took up the drug at similar rates and by 120 minutes there was no significant difference in the amount of MIL inside the two WTs. Extrapolating the graph further would suggest that WT parasites were unable to control the influx of MIL within the cell whereas MIL-R parasites were actively able to prevent the accumulation of MIL inside the cell.

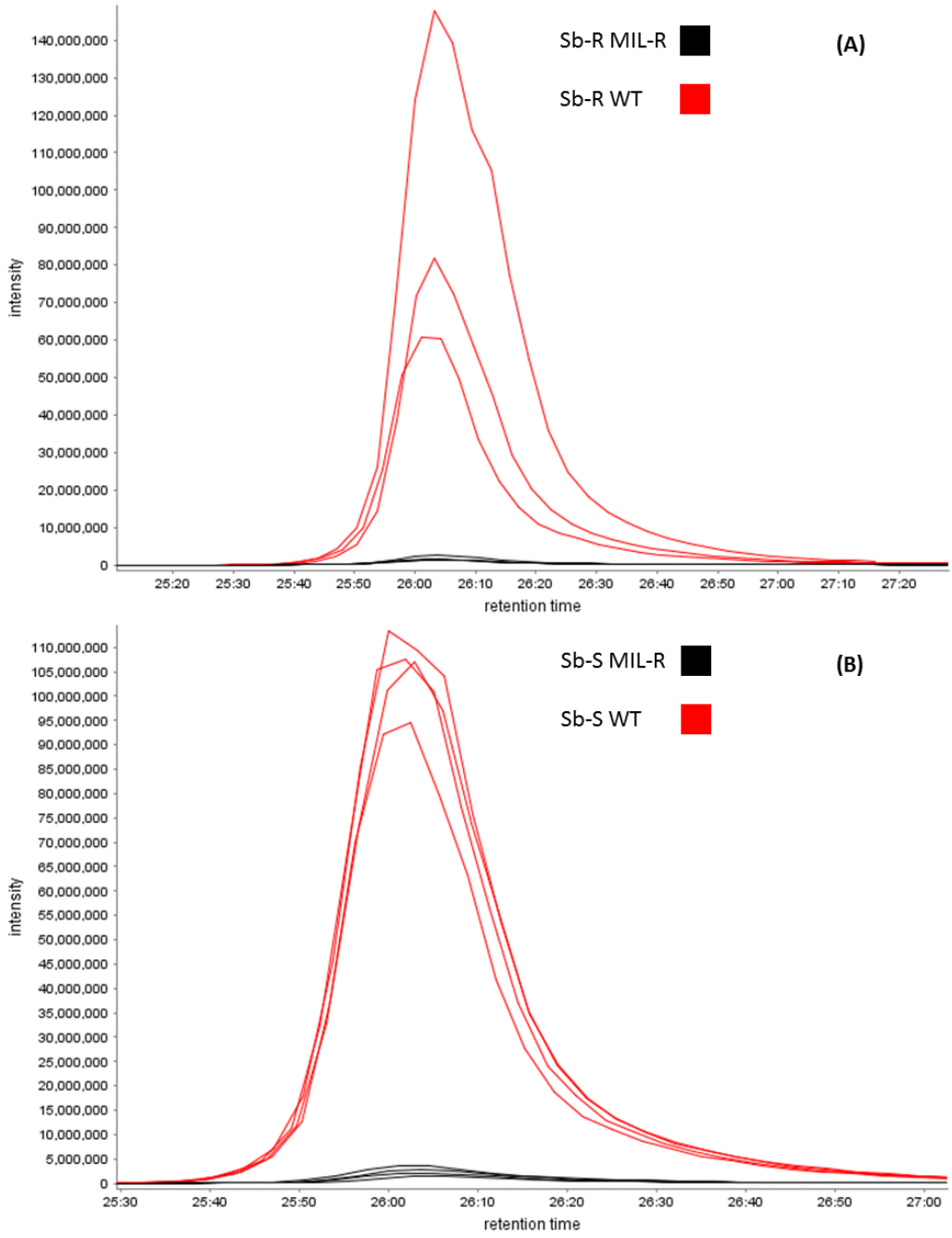


Figure 5.4. Chromatograms depicting the relative intensity of miltefosine peaks (exact mass of 407.316), extracted from the lipidome of MIL-R (black) and WT (red) promastigotes after 20 minutes of incubation with miltefosine. A) Sb-R WT and MIL-R, B) Sb-S WT and MIL-R. Chromatograms are representative of a single experiment with n = 4 technical replicates. The experiment was carried out in triplicate. Both peak differences $p < 0.001$.

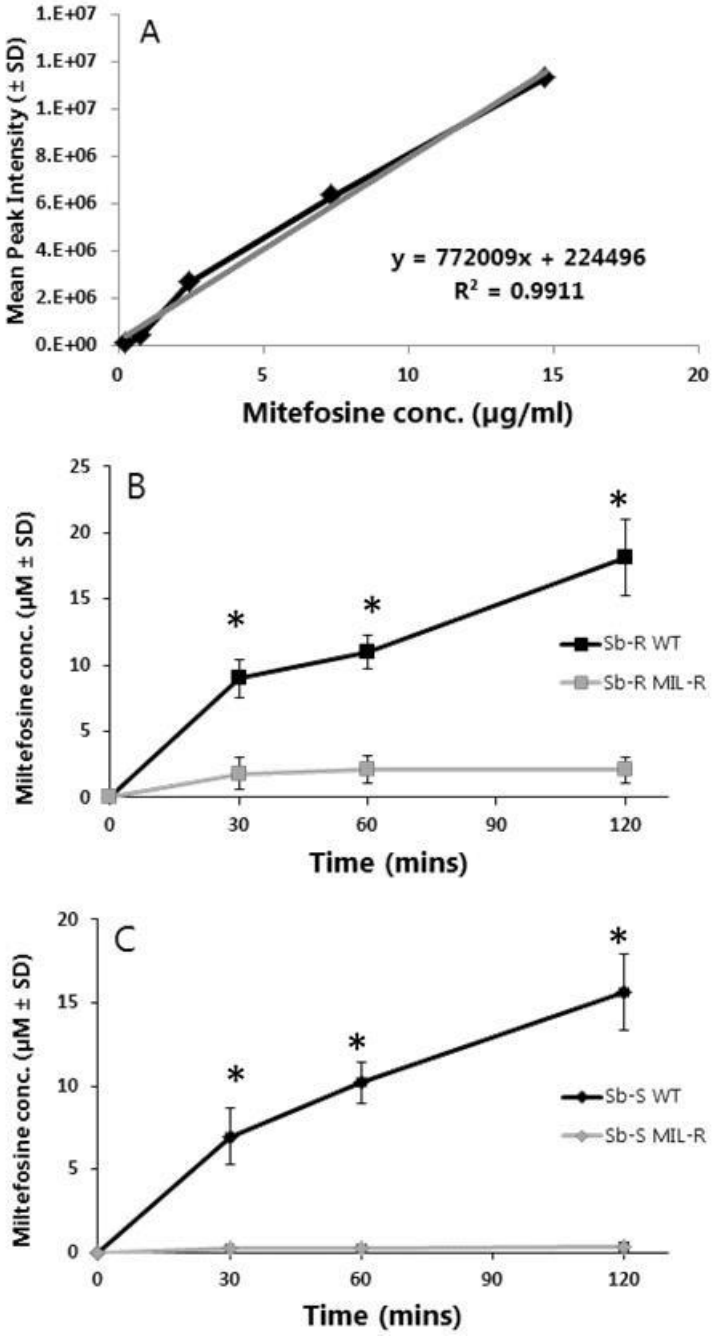


Figure 5.5. (A) Calibration curve used to quantify the MIL concentration from observed relative intensities of MIL peak (n = 3/treatment). The figure is example of the calibration curve from one of the three separate time course studies to the concentration of MIL inside of Sb-R WT (Fig. 5.6B) and MIL-R Sb-R WT and Sb-S MIL-R (Figure 5.5C). * $p < 0.05$ between MIL-R and WT.

The decreased accumulation of MIL inside MIL-R parasites has been strongly correlated to disruption in the coding sequence for the LdMT transporter in several studies, indicating that this protein is important in MIL uptake. Therefore differences in the coding sequence for the LdMT gene locus on chr13 (LdBPK_131590) was determined for MIL-R and WT by genome sequencing and PCR, using genomic and cDNA. This enabled us to determine differences at the gene level and also at and expression levels. Analysing mutations within chr13 genomic DNA revealed that two separate mutations were present in the LdMT gene of Sb-S MIL-R and one mutation in Sb-R MIL-R. A deletion (ΔLdMT) and a SNP (P691) were observed in Sb-S MIL-R. The deletion, 8.6 kb in total, spanned position 621,000 to 629,860 and covered the entirety of the LdMT gene, which is found at position 622,408 to 625,701. The deletion was first detected at 3 µM MIL selection at a frequency of 7% of the population (Figure 5.6) and gradually increased to 71% of the population at 35 µM MIL induction. Between 35 µM and 61 µM MIL however it decreased to a final frequency of 29%. The P691 SNP did not appear until 12 µM MIL induction at a frequency of 16% and had a final frequency of 67% at 61 µM MIL. Further passaging of Sb-S MIL-R in the absence of MIL, even just for 2 weeks, increased the frequency of the ΔLdMT deletion over the P691 SNP, perhaps indicating that the frequency is dependent on environmental conditions

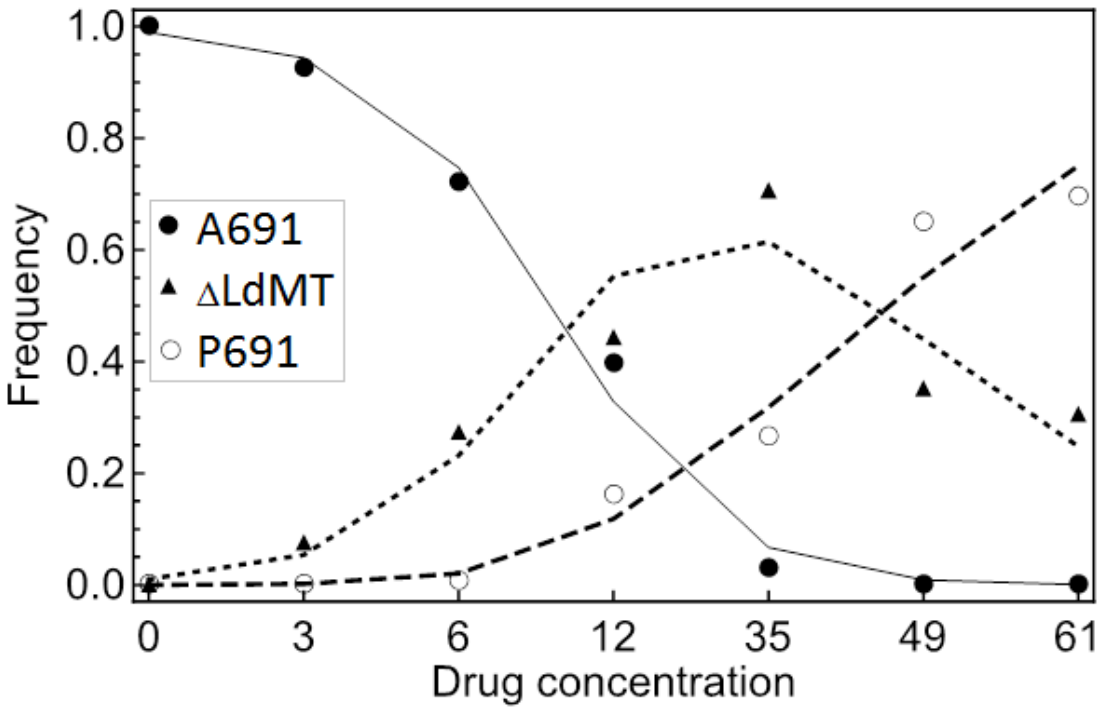


Figure 5.6. Observed and inferred frequencies for allele frequency changes (y-axis) in the LdMT gene (LdBPK_131590) in response to the drug (μM). Observed frequencies are shown for the A691, ΔLdMT, and P691 variants as solid circles, triangles, and open circles respectively. Corresponding inferences are shown as solid, dotted, and dashed lines respectively. Figure courtesy of Dr. Tim Downing and used with permission. Taken from (Shaw et al., 2016)

The deletion of the LdMT gene was confirmed via PCR using genomic DNA isolated from Sb-S and Sb-R WTs and MIL-R clones (Figure 5.7A). A PCR product for LdMT was detected for Sb-R WT, Sb-R MIL-R and Sb-S WT but not for Sb-S MIL-R. Complementary DNA was then prepared from Sb-R WT and MIL-R RNA and analysed using qPCR to determine if the mutation of the LdMT gene observed in Sb-R MIL-R had an impact upon the transcription of LdMT RNA. Transcripts for the alpha tubulin house-keeping gene were present in both samples but a transcript for the LdMT transporter gene was only found for the Sb-R WT and not in MIL-R (Figure 5.7B). Ct transcript values for Sb-R WT, Sb-R MIL-R and negative controls, respectively were; (i) for alpha-tubulin: 24.07 ± 0.28; 27.19 ± 0.84; 35.01± 0.94 and (ii) for LdMT: 27.37± 0.36; >35; >35. To further confirm a defect in the LdMT gene of Sb-R MIL-R, expression of LdROS, the beta-subunit of the LdMT was also analysed (Figure 5.7B). Transcription of LdROS in Sb-R MIL-R was significantly downregulated ($p < 0.001$) compared to WT, confirming that the expression of both the LdMT and LdROS proteins in Sb-R MIL-R was compromised.

Further analysis in differences in the genome for WTs and MIL-R showed that the copy number for chr13, which contains the LdMT gene, decreased in both strains during MIL induction. Both clones became disomic for chr13 compared to the trisomic state of WT isolates (data not shown). This occurred early in the induction of MIL resistance; at 3 µM for Sb-S and 6 µM for Sb-R MIL-R. The aneuploidy of chr13 remained disomic even after the removal of MIL pressure for two weeks.

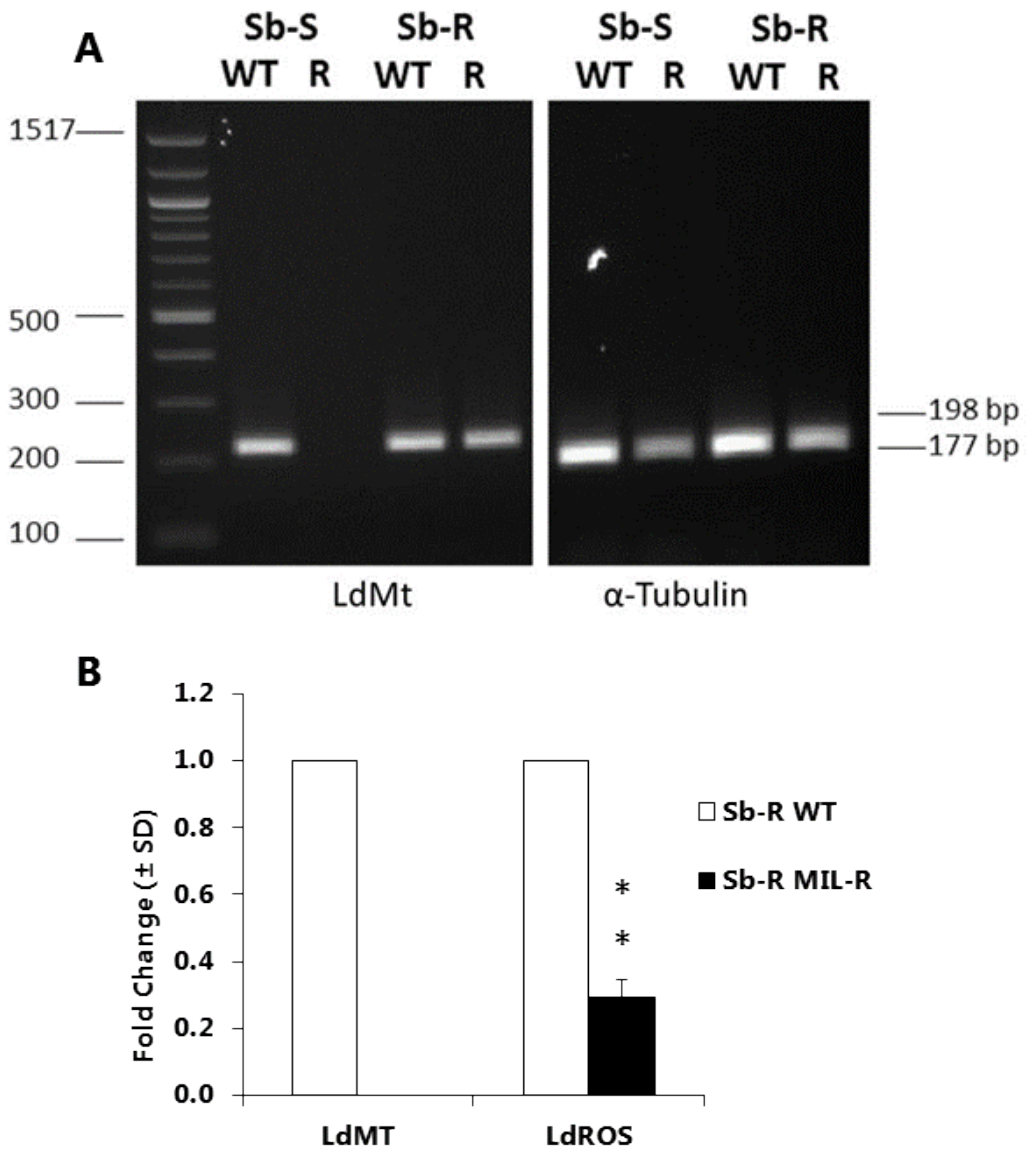


Figure 5.7. A) Agarose gel picture of PCR products amplified using LdMT and α-Tubulin primers and genomic DNA extracted from Sb-S and Sb-R WTs and their MIL-R counterparts. B) qPCR of LdMT and LdROS cDNA of Sb-R WR and MIL-R from day four promastigotes. * $p < 0.05$

5.3 Discussion

Resistance to MIL was selected for in WT Sb-S and Sb-R isolates at similar rates and resulted in comparable MIL IC_{50} values (Chapter 3). Using a genomic and metabolomic approach we uncovered that the mechanism(s) responsible for selected resistance in our two MIL-R strains developed in two distinct manners. Both MIL-R clones exhibited significant changes in their genome, metabolome and lipidome compared to their WT parents (summarised in Figure 5.10). The Sb-S MIL-R had a greater number of metabolite and lipid alterations compared to its WT, while Sb-R MIL-R exhibited a greater level of genomic change. Alterations in the LdMT gene were a feature of both MIL-R clones but the actual changes present were distinctly different. Previous studies have already shown a link between LdMT deletions or malfunctions and MIL resistance (Perez-Victoria *et al.*, 2003b, Perez-Victoria *et al.*, 2006, Perez-Victoria *et al.*, 2003a). This study highlights how these changes occurred in a longitudinal study as MIL pressure was increased and furthermore that the mutations observed in our two clones were markedly diverse and not related. This implies that there is not one common and predictable mechanism for how the LdMT gene will mutate under MIL pressure.

Changes in the genome of both MIL-R strains occurred at the very beginning of MIL selection of 3 μM MIL. This was before there was any detectable tolerance towards MIL, as the IC_{50} of WT and MIL-R parasites was comparable. The most significant change was a decrease in chromosome copy number for chr13 and chr33 that occurred in both Sb-S MIL-R and Sb-R MIL-R parasites. Aneuploidy is known to be a general stress response that has been associated with drug resistance in several

Leishmania spp. including *L. major*, *L. infantum* and *L. donovani* (Mannaert *et al.*, 2012, Kumar *et al.*, 2013, Ubeda *et al.*, 2008). Interestingly, the changes in copy number were observed at 3-6 µM MIL but a significant increase in the IC_{50} of intra-macrophages amastigotes was not observed until 12.2 µM MIL was present in the culture medium. It could be that the parasite is naturally able to withstand MIL toxicity up to this level simply by altering its chromosome copy number through aneuploidy. On the other hand, it could be that amastigotes are buffered to the effects of MIL by the host cell as the drug has to cross both the parasitophorous vacuole and the macrophage membrane, so that local concentration may be much lower than that which is present in the culture medium. Chromosome copy number varies rapidly *in vitro* and therefore aneuploidy is easily assessable to *Leishmania*. We propose that aneuploidy allows the rapid adaptation of the parasite to the external stress of MIL, allowing it to survive at intermediate drug concentrations. This transitional fitness state then permits rarer mutations to increase in frequency in the population and increase parasite fitness. This is similar to the situation where a loss of function mutation can occur, as described by (Ashley *et al.*, 2014). In our Sb-S parasites, this would be described as the aneuploidy allowing the initial adaptation to MIL at low concentrations (3-12 µM) until the LdMT mutation and P691 SNP increases in frequency at higher MIL concentrations (6-35 µM for the deletion and 35 µM and above for P691). In the Sb-R parasites, aneuploidy was observed at 3 µM and above and the LdMT SNP did not appear until 12.2 µM.

The deletion of the LdMT gene was present at a 1% frequency level in the Sb-S WT population, indicating that it already pre-existed within the population. It therefore

infers that in the absence of MIL, this deletion carries only a very minor fitness effect which then becomes a strong positive effect in the presence of MIL and so the frequency of these parasites increase. In contrast, the P961 SNP mutation was observed at a frequency of 0.02% in the WT population (Figure 5.6). This suggests that it is likely to be a *de novo* mutation. The P961 SNP rose to 67% while the ΔLdMT deletion was only found at 29% in the final MIL-R clone. This dominance of the P691 SNP over the ΔLdMT deletion indicates that the P691 mutation is more advantageous than the deletion at higher concentrations of MIL. Perhaps the point mutation preserves some of the function of the LdMT transporter for phospholipids other than MIL. This could be due to a deleterious effect of also removing a neighbouring hypothetical gene (LdBPK_131600, at 627,613-629,076) or perhaps because the ΔLdMT may possess an unknown function that is preserved in the P691 SNP. By the 35 μM MIL induction concentration, only 2% of the MIL-R population did not possess a mutated LdMT gene. Such a low frequency is indicative that at this concentration and beyond, the WT ΔLdMT was not viable. The downregulation of chr33 would also influence MIL uptake in MIL-R parasites. Five ABC transporter genes that are associated with drug influx and efflux in *L. donovani* (ABCF3, LdBPK_330340; unnamed ABC gene, LdBPK_331370; ABCD3, LdBPK_331960; ABCI3, LdBPK_333200; ABCI4, LdBPK_333410; Jeddi *et al.*, 2011, Manzano *et al.,* 2013) would also be downregulated with the reduction in chr33 copy number.

Mutations in the ΔLdMT gene of both MIL-R clones is likely to be the main reason for the significant reduction in MIL inside promastigote parasites detected using LC-MS. Sb-S and Sb-R MIL-R had 98% and 89% respectively less MIL inside their cells

after culturing in media containing MIL for 120 mins. The almost non-existent level of MIL inside MIL-R parasites is undoubtedly the major factor in the parasite's ability to survive the drug. The fact that MIL levels were extremely low in MIL-R parasites after only 30 minutes of MIL exposure, and remained at a near constant level suggest a reduction in MIL uptake rather than increased efflux. In addition, both WT isolates appeared to accumulate MIL at higher levels than was present in the culture media suggesting that they are actively uptaking the drug.

Mapping every genomic change to a specific change in the metabolome is simply not possible. In addition, single genomic changes could easily be responsible for a plethora of differences in the metabolome, for example, a change in membrane composition could alter the uptake of several metabolites by the parasite. Similarly, altering one metabolite can have a knock on effect to a range of other metabolites. Moreover, all the metabolomic pathways in *Leishmania* are not fully elucidated and how one metabolite connects to another may not correlate to those that occur in other species. Despite this, the metabolome and lipidome of MIL-R parasites can still be mapped and compared to that of WT isolates to extrapolate possible cause and effects of MIL resistance. This study showed that MIL induction resulted in significant differences in their metabolite and lipid profiles and that the effects were strain-specific.

Sb-S MIL-R parasites had a greater number of metabolite differences but with a smaller number of lipid differences compared to Sb-R MIL-R. Additionally, all of the metabolites and lipids that were altered in Sb-S MIL-R were associated with an

increase in levels in MIL-R parasites. Several of these metabolites were amino acids (leucine, valine, and serine) or purines/pyrimidines (hypoxanthine, xanthine and uridine) that were present in the culture medium and it is possible that the increase can be accounted for by an increased up take from the medium. Previous studies have reported that a modification to lipid metabolism in drug resistant *Leishmania* resulted in increased uptake of amino acids and purines/pyrimidines that were structurally unrelated to the drug the parasites were resistant to (Basselin *et al.*, 1997, t'Kindt *et al.*, 2010). This concept that altering the lipid and therefore membrane composition can indirectly alter the transport of other non-related metabolites is supported by this study.

Both MIL-R parasites exhibited significant changes in metabolites involved in lipid metabolism, specifically the Kennedy pathway. The entirety of Kennedy pathway metabolites found to be up regulated in Sb-S MIL-R were located on the choline branch of the pathway (Figure 5.8). The increase in metabolites in the choline branch correlates with the significant increase in the PC content of Sb-S MIL-R. Eleven acyl-PCs were significantly upregulated in the MIL-R parasites of Sb-S. CDP-choline was the only metabolite on the choline branch of the Kennedy pathway not to be significantly upregulated in Sb-S MIL-R. This is not surprising, as conversion of choline phosphate to CDP-choline represents the rate limiting step in the pathway, and it is tightly controlled by choline-phosphate cytidylyltransferase (Smith and Butikofer, 2010). Of the eleven PCs upregulated in Sb-S MIL-R, eight had between 1 and 4 double bonds in the fatty acid tails of their PCs. The compositions of lipids in the parasite membrane are known to have a significant effect on fluidity. Increasing

the chain length or saturation of fatty acid tails can have an impact on the resistance to oxidative stress and membrane fluidity. Both long chain and polyunsaturated fatty acid chains on phospholipids are known to alter the fluidity of membranes by reducing the order state and producing kinks in the membrane respectively. The PCs that were found to be increased in Sb-S MIL-R parasites contained polyunsaturated, long chain tails. Moreover, polyunsaturated bonds in PCs are known to protect *Leishmania* from oxidative stress which has been associated with MIL treatment (Zhang and Beverley, 2010). It is possible that the increase of polyunsaturated PCs in Sb-S in beneficial to the parasite by preventing any MIL that would be transported passively through the membrane from entering the parasite and therefore protecting itself from MIL induced toxicity. Of the two MIL-R clones, Sb-S MIL-R has the greatest reduction in MIL uptake when incubated with the drug (98% and 89% reduction respectively). This again points towards the fact that the membrane of Sb-S MIL-R could be less permeable than that of its WT and also compared to the two Sb-R lines.

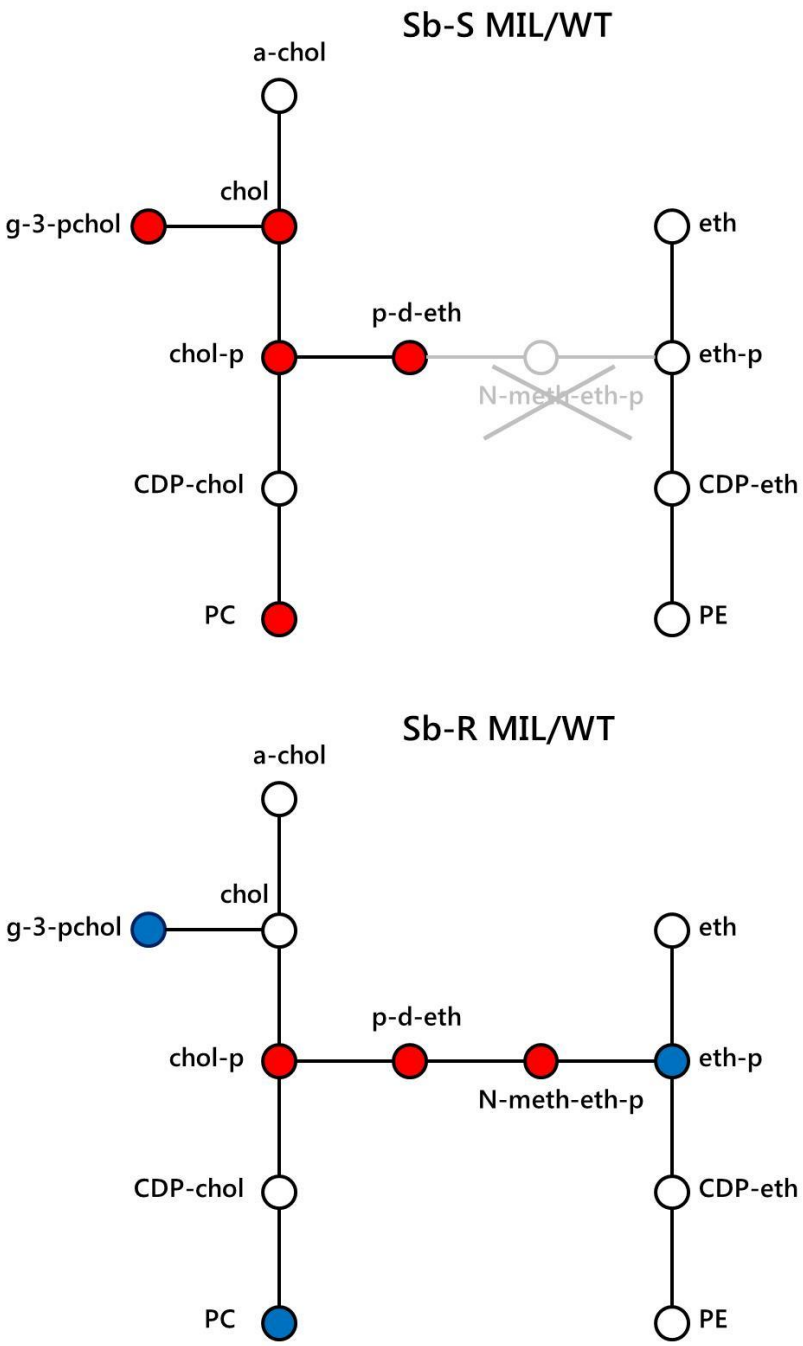


Figure 5.8 Diagrammatic representation of the Kennedy pathway metabolites altered in the MIL-R parasites of Sb-S (A) and Sb-R (B) compared to their WT. A blue circle denotes a significant down regulation ($p < 0.05$) of the metabolite in the MIL-R strain compared to its WT. A red circle denotes a significant up regulation ($p < 0.05$) in the MIL-R. Key: a-chol, acytelcholine; g-3-pchol, glycero-3-phosphocholine; chol, choline; CDP-choline, cytidine diphosphate-choline; PC, phosphatidylcholine; p-d-eth, phosphodimethylethanolamine; N-meth-eth-p, N-methylethanolamine phosphate; eth, ethanolamine; eth-p, ethanolamine phosphate; CDP-eth, cytidine diphosphate-ethanolamine; PE, phosphodimethyethanolamine.

In contrast to Sb-S MIL-R, Sb-R MIL-R had alterations to both branches of the Kennedy pathway, several of which metabolites that were down regulated (Figure 5.8 and Figure 10). Changes in the Sb-R MIL-R clones appear to be centred on the ethanolamine branch of the Kennedy pathway and more specifically, production and metabolism of ethanolamine phosphate. This metabolite is the second step of the ethanolamine branch of the Kennedy pathway, responsible for the production of PEs and sphingomyelins in *Leishmania*. Ethanolamine phosphate is synthesised *de novo* in *Leishmania* either via the phosphorylation of scavenged ethanolamine or as a product of sphingolipid metabolism (Figure 5.9) that starts with the scavenging of serine and incorporates sphinganine, ceramides, SMs and sphingosine (Smith and Butikofer, 2010). Ethanolamine phosphate was down regulated by a factor of 5 in Sb-R MIL-R as was the majority of the sphingolipid pathway. The only exception was that serine, the original precursor of the whole pathway, was found significantly upregulated. Palmitoyl Co-A which incorporates serine to form 3-dehydro-sphinganine, was not resolved by LC-MS. Given that it was not resolved in any sample of any isolate across all of our experiments it is likely that its large size (1005.34 m/z) places it outside the limits of detection for the method used. Ethanolamine was not supplemented in the culture medium used and so the only mechanism for ethanolamine phosphate production would be from the sphingolipid pathway. Surprisingly, despite the significant decrease in ethanolamine phosphate in Sb-R MIL-R, the levels of PE appeared to be unaffected and it was the PC content that was significantly downregulated in Sb-R MIL-R. Closer analysis of the lipid anchors of PEs revealed that while the total PE content of Sb-R MIL-R was similar to

WT, a significant alteration in the lipid anchor classifications existed. Specifically, MIL-R clones of Sb-R had a marked decrease in the proportion of 1-alkyl-2-acyl PEs and an increase in di-acyl- and 1-alkenyl-2-acyl- PEs compared to WT. We hypothesise that although the amount of PEs present in the total lipid pool is similar between Sb-R WT and MIL-R, the synthesis pathway has changed. PEs are synthesised *de novo* in *Leishmania* via three different mechanisms; 1) Via reversible head group exchange with PC or PS, 2) From the Kennedy pathway and 3) Via the decarboxylation of PS reviewed (Zhang and Beverley, 2010). Previous data and results from this study confirm that PS is absent from the promastigote stage of both *L. donovani* and *L. major*, thus ruling out the headgroup exchange or decarboxylation with PS as a precursor of PE synthesis in our strains (Weingärtner *et al.*, 2012). Moreover, Sb-R MIL-R had significantly reduced levels of sphingoid bases and SLs which are a known source of ethanolamine phosphate in *Leishmania*. This was ultimately reflected in the significantly reduced levels of ethanolamine phosphate in Sb-R MIL-R. We therefore hypothesise that in the Sb-R MIL-R clones, the reduced levels of ethanolamine phosphate forces the parasite to synthesise PE via an alternative source, namely the base exchange of PCs to form PEs. This would explain the observed reduction in diacyl-PCs and the increase in proportion of PEs with diacyl anchors in the PE pool of Sb-R MIL-R compared to WT. The PCs that were observed to be downregulated in Sb-R MIL-R all had diacyl-lipid anchors. Exchanging the head group of a diacyl-PE would therefore translate to a PE that also has a diacyl lipid anchor. PE is essential to *Leishmania* and it therefore stands to reason that healthy levels of the metabolite would need to be maintained. Previous

studies utilising RNAi on ethanolamine phosphotransferase in *Trypanosoma* would also support this hypothesis (Signorell *et al.*, 2008). Conditional knockdown of ethanolamine phosphotransferase elucidated that choline phosphotransferase could compensate for the silenced ethanolamine phosphotransferase. As in our study, the PE content of *T. brucei* changed from the predominantly alk-1-enyl-acyl PE to diacyl.

As explained in the previous chapter (Chapter 4), our detection and identification methods are not infallible. Resolving the exact structure of certain lipids, mainly SMs, is not possible. SMs are sphingolipids composed of a ceramide, a fatty acid tail and a polar head group which is either a choline or ethanolamine moiety. Due to the nature of their chemistry and the fact that different SMs can have the same mass, the head group identity cannot be ascertained without fragmentation. For example, SM (33:1) [containing a phosphocholine] would have the exact same mass and likely be eluted at the same time as PE-Cer (36:1) [containing phosphoethanolamine]. These SMs are clearly two distinctly different species but resolving the identity using standard LC-MS would be impossible. Fragmentation would allow the head group and tails to be broken apart and identified more specifically. It is therefore possible that our parasites have changed the composition of the headgroup of their SMs in response to MIL induction or that changes in one class of SMs are being masked by that of another class that resolve at the same mass. It is however certain that Sb-R MIL-R has a significant reduction in the total content of SMs compared to WT. The nature of this decrease cannot be known for definite but the fact that it is reducing is clear.

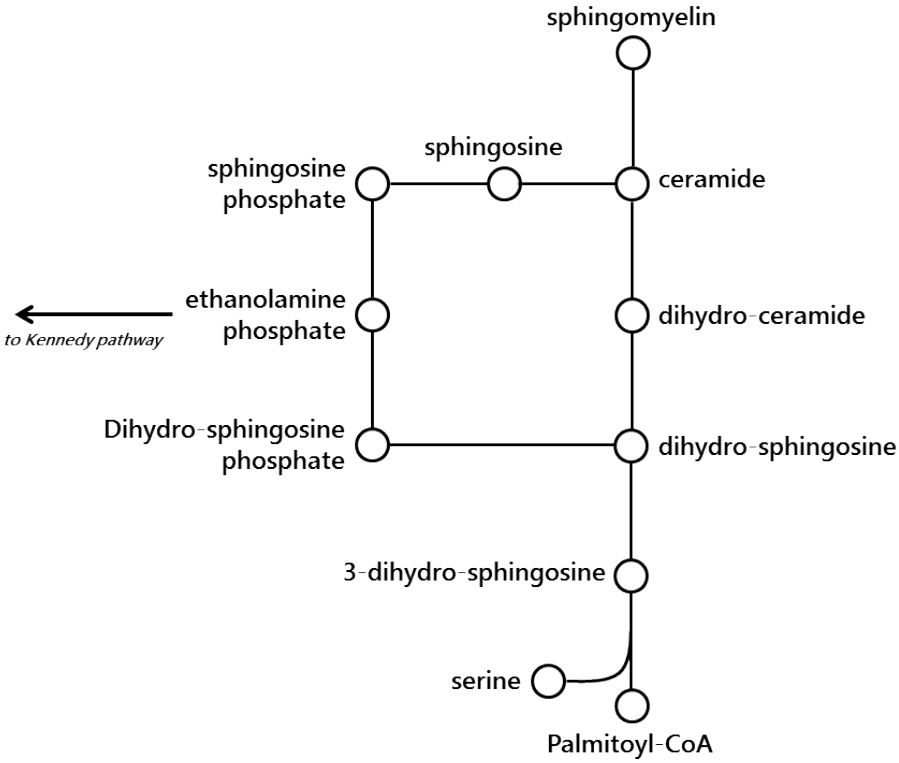


Figure 5.9. Sphingolipid biosynthesis and metabolism in *L. donovani*. Created using Microsoft excel based on information from sphingolipid metabolism pathway found on Kegg database (http://www.kegg.jp/kegg-bin/highlight_pathway?scale=1.0&map=ldo00600&keyword=sphingosine).

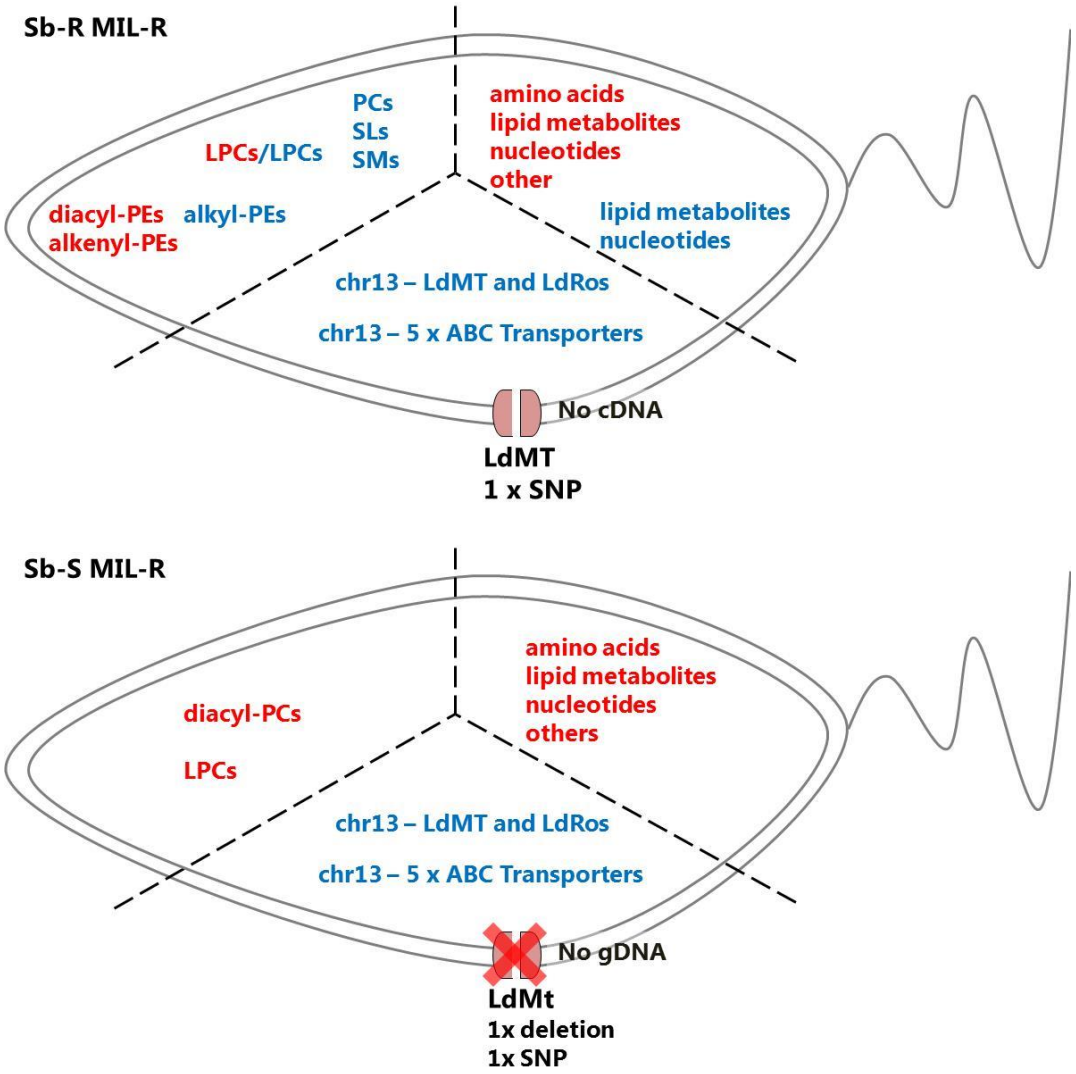


Figure 5. 10. Graphical representation of the changes in MIL-R promastigotes compared to WT after selection of MIL resistance.

In summary, it would appear that while both our MIL-R clones share certain similarities in their paths to MIL resistance, the overall journeys are markedly different. As seems to be the case with all lab derived MIL-R *L. donovani*, our MIL-R lines had substantial alterations to the LdMT and LdROS genes, responsible for the main transporter of the drug in to the cell. How this occurred however appears to be case specific as in one case a large deletion and in the other, multiple SNPs were responsible for the lack of gene transcription. Genetically, both MIL-R clones appear to cope with MIL pressure via aneuploidy as a buffer to allow more specific mutations and deletions to dominate. Similarly, the frequency of LdMT mutations rises in the populations along with a number of other genetic alterations that incorporated genes known to be involved in drug transport. Moreover, the lipid content of MIL-R parasites underwent large scale alterations in drug resistant parasites. Sb-S MIL-R appeared to significantly upregulate lipid production, most notably in long chain, unsaturated PCs. This is likely to have decreased the permeability of the parasite membrane, a hypothesis that is somewhat vindicated by the near total exclusion of MIL from inside the cell of Sb-S MIL-R promastigotes. On the other hand, Sb-R MIL-R had a significant decrease in a variety of lipids. Unlike in Sb-S MIL-R, it appears that the changes in Sb-R MIL-R were not advantageous to the exclusion of MIL but more likely to be as a consequence of a defect in SL metabolism. The reorganisation of the SL and PC repertoire of the cell look as if it is a trade-off of maintaining the PE levels within the cell. Once again, it should be stressed that these studies were performed on promastigotes so how they translate to amastigotes is unknown. What is clear however is that the

mechanism of MIL resistance cannot be predicted and that tolerance to the drug can be achieved in a number of different ways. This could therefore complicate the efforts of predicting the likelihood of MIL resistance occurring in endemic regions and also management strategies for how to avoid resistance. A singular mechanism for resistance would allow strategies to be implemented and managed relatively easily. What, if any, consequences these mechanisms of MIL resistance have on the fitness of parasites are also of interest and will be looked at in the next chapter.

In conclusion, MIL-R parasites were compared to their WT counterparts to establish a correlation between selection of drug resistance and changes in the metabolome and lipidome of promastigotes. The main conclusions from the work were:

- Selection of MIL resistance in both Sb-R and Sb-S was associated with a decreased accumulation of MIL inside promastigotes. In both cases this correlated with a disruption to the LdMT gene resulting in either a lack of gDNA or transcription of RNA in MIL-R parasites
- The copy number of chromosome 13, that contains genes for the LdMt and a number of other drug transporters, was decreased in both MIL-R strains compared to their WT.
- Although both MIL-R lines shared some homology in their resistance, the mechanisms/implication of resistance appears to be very different.
- Sb-S MIL-R was associated with a significant increase in a number of metabolites and lipids, particularly PCs.

- Conversely, Sb-R MIL-R was associated with a significant decrease in PCs and a large scale reorganisation of lipid synthesis and metabolism.

Chapter 6. Fitness of *L. donovani* Selected for Resistance to Paromomycin or Miltefosine

6.1 Introduction

Parasites resistant to MIL and PMM were generated to study the genetic and metabolomic effects associated with drug resistance. Another important consideration is what impact drug resistance may have on the ability of the parasites to infect and survive within their host.

Upon infection via a sandfly, *Leishmania* parasites encounter a range of cell killing mechanisms mediated mainly by the innate immune system of the host. The interaction of macrophages and neutrophils and in particular the production of superoxide and TNF-α is critical in the elimination of *Leishmania* (Mougneau *et al.*, 2011). Numerous cytokines also play an important role in signalling to immune cells and activation of specific immune responses such as switching of cells to Th1 responses. IL-10 and IL-12 in particular play a crucial role in the activation of CD4+ cells that, along with NK cells and CD8+ T cells, produce IFN-γ, an important molecule in parasite killing (Prajeeth *et al.*, 2011).

Any genetic or metabolomic changes between WT and drug resistant parasites have the potential to have an adverse or positive effect on parasite survival by altering their ability to infect, proliferate and survive within its host. Any fitness cost or gain will impact upon the dominance of drug resistant parasites in the endemic population. For example, if drug resistance is associated with a negative fitness cost then resistant parasites will only proliferate in a population when said parasites are under the selective pressure of the given drug. Without the selective pressure of the drug, parasites with a fitness deficit are unlikely to proliferate. On the other

hand, if resistance to a drug is associated with an overall fitness benefit compared to susceptible parasites then resistant parasites will likely persist and potentially out-compete other less fit parasites, regardless of external drug pressure. Resistance to Sb in the ISC is still prevalent despite the drug being abandoned as a treatment option in the endemic area (Ready, 2014). This suggests that parasites resistant to Sb have maintained a fitness advantage, or at least a neutral fitness cost, over Sb susceptible parasites, even when drug pressure has not been a factor. Several studies have in fact reported that Sb-resistant parasites have a greater tolerance to macrophage killing effects, such as oxidative stress via ROIs, over Sb susceptible parasites (Carter *et al.*, 2005, Guha *et al.*, 2014, Mukhopadhyay *et al.*, 2011).

MIL resistance is easily selected in laboratory models and this has led to the assumption that resistance emerging in the field is on a matter of time and indeed, relapses of patients treated with MIL have been reported (Dorlo et al., 2012, Rijal et al., 2013). Despite this, parasites taken from relapse patients in the ISC do not show a decreased susceptibility towards MIL (Bhandari *et al.*, 2012, Rijal *et al.*, 2013). To date, the only clinical isolates from treatment failures that also show a resistance to MIL have been isolated from patients suffering from HIV co-infections (Cojean *et al.*, 2012). A recent study analysing the effect of MIL resistance on the fitness of MIL-R *L. major* found that while resistant parasites had elevated metacyclogenesis and therefore an increased number of infective metacyclic promastigotes, their virulence both *in vivo* and *in vitro* was significantly reduced (Turner *et al.*, 2015). Hendrickx *et al.* attempted to generate MIL-R parasites *in vivo* using a Syrian

hamster model that was subjected to numerous cycles of treatment and relapse. After 5 cycles of treatment and relapse, MIL-R parasites could not be generated. In contrast, PMM-R amastigotes were successfully generated after only three treatment and relapse cycles (Hendrickx *et al.*, 2014). Together, these findings raise questions about how readily MIL resistance can be spread in an endemic population and how well the *in vitro* data of easily selected MIL resistance can be transferred to the field.

As mentioned above, PMM-R amastigotes can be generated *in vivo* using Syrian hamsters and cycles of treatment and relapse (Hendrickx *et al.*, 2014). Promastigote resistance to PMM can also be easily selected for *in vitro* (Berg *et al.*, 2015). In contrast to MIL-R, PMM-R parasites have been reported to have an increased fitness profile when compared to WT and moreover, this fitness was also present in parasites resistant to combinations of drugs that included PMM (García-Hernández *et al.*, 2015). Interestingly, PMM-R lines generated by Bhandari *et al.* displayed characteristics similar to what is observed is Sb-R parasites, namely increased resistance to; nitric oxide stress, SIN, SNAP and IFN-γ as well as increasing the stimulation of host IL-10 (Bhandari *et al.*, 2014). PMM-R parasites may therefore have fitness advantages over PPM susceptible parasites in the endemic population which could have large implications for the use of the drug in combatting VL.

Both PMM and MIL have been shown to induce NO and TNF-α production in drug treated macrophages via the action of toll-like receptor 4 (Das *et al.*, 2012). MIL treatment has also been associated with an increase in IFN-γ levels (Wadhone *et al.*,

2009). Therefore resistance to MIL or PMM could also be associated with increased resistance to host cell killing mechanisms in *Leishmania*. If this is the case then emergent drug resistant strains could be better adapted to survive within the host even in the absence of drug pressure. If this was to be the case then the proliferation of drug resistant parasites in the endemic population could remain high. This could have serious implications on the clinical life of these drugs in *Leishmania* endemic areas and it may impact on how these drugs are used within these areas. For these reasons, we sought to characterise the fitness of our drug resistant parasites in terms of how well they could both infect macrophages and survive various macrophage killing mechanisms. Another factor when considering the fitness of the parasite is the ability to be transmitted both to and from the sandfly. Unfortunately, due to time constraints and lack of direct access to sandflies, this criterion of fitness was not assessed in this study.

Therefore the main objectives of this study were to assess:

1) The ability of MIL-S and MIL-R *L. donovani* promastigotes to infect macrophages and their susceptibility to macrophage products (NO and SNAP) and change into metacyclic parasites.

2) Compare the ability of PMM-S and PMM-R *L. donovani* promastigotes to infect macrophages and their susceptibility to macrophage products (NO and SNAP) and change into metacyclic parasites.

3) Determine if drug resistance (MIL or PMM) of *L. donovani* had any effect on cytokine (IL-6, IL-10, IL-12 or TNF alpha) or nitric oxide (measured indirectly using nitrite) production by infected macrophages.

6.2. Results

6.2.1 Fitness of MIL-R parasites

MIL-S and MIL-R *L donovani* promastigotes were cultured *in vitro* with different concentrations of SIN, a donor of both nitric oxide and superoxide; or SNAP, a NO donor, to determine their susceptibility of different microbial products released by stimulated macrophages parasites. Sb-S MIL-R promastigotes were 2 times more susceptible to SNAP than their Sb-S WT counterparts ($p < 0.05$ Table 6.1). In contrast there was no significant difference in the susceptibility of Sb-R MIL-R promastigotes compared to Sb-R WT with SNAP treatment (Table 6.1). Neither was there a difference in the susceptibility of Sb-S WT and MIL-R to SIN treatment however Sb-R MIL-R parasites were significantly resistant to the effects of SIN compared to their WT ($p < 0.05$ Table 6.1). The IC_{50} of SIN treatment on Sb-R MIL-R of 466 µM was significantly greater than 313 µM of it WT counterpart. Promastigotes were also treated with human serum as a measure of metacyclogenesis. Neither of the MIL-R clones exhibited any significant difference in their susceptibility to killing after exposure to human serum, suggesting that both MIL-R parasites were transforming in to metacyclics at the same rate as WT (Table 6.1)

The effect of MIL resistance on the ability of parasites to infect and survive within macrophages was determined by comparing infection levels at 96 hours post-infection. In addition, the susceptibility of intracellular amastigotes to NO and ROS were assessed by treating infected macrophages with different concentrations of

SIN or SNAP. However, the infectivity of Sb-S MIL-R was consistently lower than that of its WT and often at levels below 30% infectivity which made collecting reliable data difficult. This indicated that MIL resistance was having a detrimental impact on Sb-R infectivity. Indeed, Sb-R MIL-R amastigotes were significantly more susceptible than Sb-R WT to SNAP treatment (Table 6.1) however MIL resistance had no significant effect on the susceptibility of Sb-S compared to its WT. The effect of SIN on intracellular amastigotes was not tested as it was found to be toxic to host macrophages.

Bone marrow derived mouse macrophages infected with intracellular amastigotes were also stimulated with IFN-γ and LPS to determine if selected resistance to MIL influenced the cytokines produced by infected macrophages. A dose dependent reduction in parasite survival occurred for IFN-γ and LPS treated macrophages was observed at both concentrations (Figure 6.1). Neither Sb-S MIL-R nor Sb-R MIL-R differed significantly from their WT in terms of percentage survival at each IFN-γ and LPS concentration. Macrophages infected with *L. donovani* produced significantly less IL-10 when stimulated with IFN-γ and LPS compared to stimulated uninfected macrophages ($p < 0.001$, Tables 6.2.A and 6.2.B). IL-12 production in macrophages infected with *L. donovani* was also significantly impaired compared to uninfected macrophages ($p < 0.05$). Importantly, both Sb-S strains suppressed IL-12 production at an even greater level than their Sb-S MIL-R counterparts ($p < 0.05$, Table 6.2.A). Production of nitrite by uninfected and infected macrophages was induced by IFN-γ and LPS treatment. Importantly, the amount of nitrite produced by activated macrophages was only inhibited (compared to uninfected controls) in cells

infected with Sb-R MIL-R treated with the lower dose of IFN-γ and LPS ($p < 0.05$, Table 6.2.B). Nitrite production in Sb-S infected macrophages was only supressed by Sb-S WT at the higher dose of 100:100 ng:ml IFN-γ and LPS ($p < 0.05$ Table 6.2.A). Stimulation of infected and uninfected macrophages produced a significant production of TNF-α compared to unstimulated cells however there was no significant difference in the levels produced between infected and uninfected macrophages. Unfortunately, in some experiments, TNF-α production was variable, indicating that the ELISA method was not reliable. This could reflect the batch of antibodies used in these studies or the fact that cytokines were not determined immediately after the experiment.

Table 6.1. The effect of SIN, SNAP and human serum treatment on the survival of WT and MIL-R promastigotes and intracellular amastigotes. IC50 values are from three separate experiments. *$p < 0.05$ comparing respective WT and MIL-R clone.

	Mean IC_{50} value ± SD			
	SIN (μM)	**SNAP (μM)**		**Human Serum (μl)**
	Promastigote	**Promastigote**	**Amastigote**	**Promastigote**
Sb-S WT	346 ± 47	117 ± 14	1242 ± 62	3.6 ± 1.1
Sb- S MIL-R	363 ± 60	55 ± 5*	1062 ± 131	3.5 ± 1.2
Sb-R WT	313 ± 29	57 ± 8	934 ± 41	4.3 ± 1.6
Sb- R MIL-R	466 ±88*	71 ± 4	576 ± 68*	2.9 + 1.0

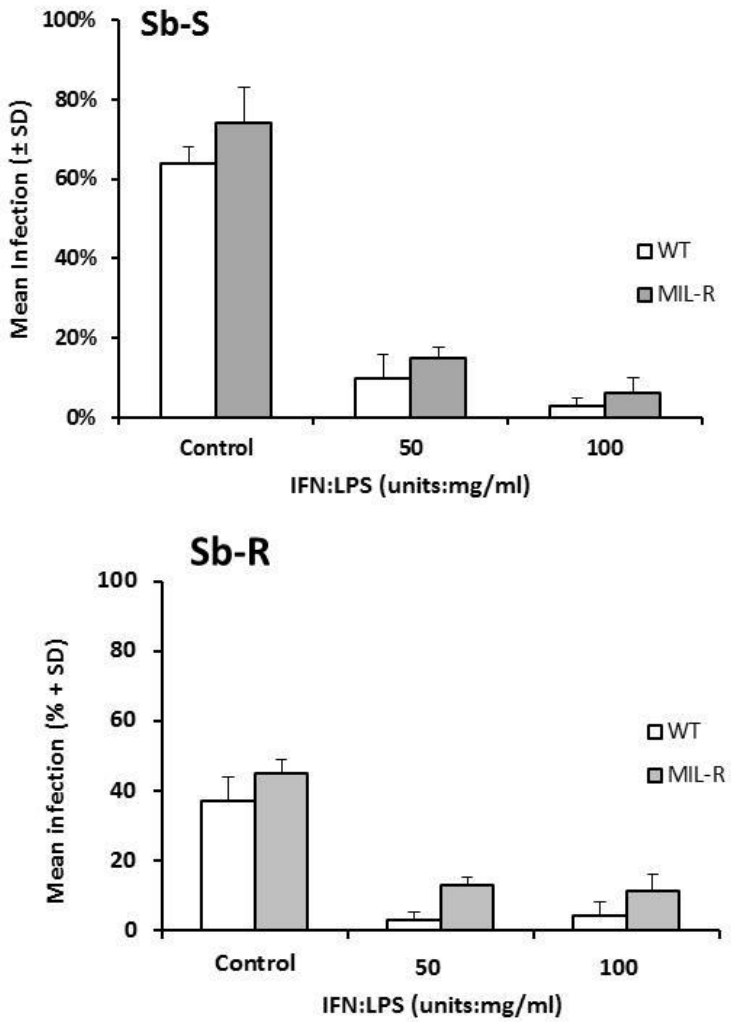


Figure 6.1. The effect of IFNγ/LPS treatment on the *in* vitro survival or WT and MIL-R *L. donovani.* Macrophages (0.5 x 10^5) were infected on day 0 with 2x10^6 promastigotes. Parasite infected macrophages were incubated with IFNγ/LPS for 72 hours and the infectivity of parasites determined by counting Giemsa stained slides. No significant difference between the infectivity of WT and MIL-R clone parasites was found.

Table 6.2. Concentration of cytokines produced by uninfected macrophages and macrophages infected with; A (Sb-S WT and MIL-R) and B (Sb-R WT and MIL-R) intracellular amastigotes stimulated with IFN-γ and LPS.

A		**Cytokine (ng/ml ± SD)/nitrite level (µM ± SD)**		
		Control	**IFN/LPS (50:50)**	**IFN/LPS (100:100)**
IL-10	Uninfected	0.07 ± 0.05	0.48 ± 0.07**	1.53 ± 0.27***[a]
	Sb-S WT	0.17 ± 0.11	0.09 ± 0.03	0.06 ± 0.10
	Sb-S MIL-R	0.05 ± 0.05	0.14 ± 0.14	0.14 ± 0.01
IL-12	Uninfected	0.06 ± 0.05	1.02 ± 0.25**	0.97 ± 0.16**
	Sb-S WT	0.0 ± 0.0	0.03 ± 0.03	0.06 ± 0.03
	Sb-S MIL-R	0.0 ± 0.0	0.05 ± 0.05	0.25 ± 0.04*[a]
TNF alpha	Uninfected	0.51 ± 0.38	0.89 ± 0.45	3.59 ± 0.32
	Sb-S WT	0 ± 0	1.33 ± 0.60	3.44 ± 0.09
	Sb-S MIL-R	0 ± 0	0.45 ± 0.45	3.93 ± 0.39
Nitrite	Uninfected	3.8 ± 2.2	14.9 ± 2.3**	18.6 ± 3.5**
	Sb-S WT	6.3 ± 0.7	19.7 ± 4.1*	12.6 ± 4.1**[a]
	Sb-S MIL-R	4.1 ± 1.8	22.8 ± 2.8***	19.8 ± 5.6**

B		**Cytokine (ng/ml ± SD)/nitrite level (µM ± SD)**		
		Control	**IFN/LPS (50:50)**	**IFN/LPS (100:100)**
IL-10	Uninfected	0.07 ± 0.05	0.48 ± 0.07**	1.53 ± 0.27***[a]
	Sb-R WT	0.16 ± 0.02	0.14 ± 0.01	0.14 ± 0.02
	Sb-R MIL-R	0.17 ± 0.01	0.17 ± 0.03	0.15 ± 0.02
IL-12	Uninfected	0.06 ± 0.05	1.02 ± 0.25**	0.97 ± 0.16**
	Sb-R WT	0.16 ± 0.02	0.14 ± 0.01	0.14 ± 0.02
	Sb-R MIL-R	0.01 ± 0.03	0.03 ± 0.04	0.08 ± 0.01
TNF alpha	Uninfected	0.51 ± 0.38	0.89 ± 0.45	3.59 ± 0.32
	Sb-R WT	2.50 ± 0.02	3.52 ± 0.05	0.21 ± 0.03
	Sb-R MIL-R	1.01 ± 0.43	2.55 ± 0.25	2.65 ± 0.16
Nitrite	Uninfected	3.8 ± 2.2	14.9 ± 2.3**	18.6 ± 3.5**
	Sb-R WT	4.8 ± 1.8	3.2 ± 0.2	21.3 ± 1.1***[a]
	Sb-R MIL-R	6.2 ± 1.4	4.7 ± 4.0	16.1 ± 4.7*

6.2.3 Fitness of PMM-R parasites

Similar studies to those described above for MIL were carried out using PPM-R parasites derived from the same WT parents plus the additional Sb-I WT and PMM-R. Paromomycin resistant promastigotes and intracellular amastigotes were likewise screened for their susceptibility to various cell killing mechanisms including SIN, SNAP, IFN-γ and LPS. The Sb-S WT parent was found to be naturally resistant to PMM despite the fact that the patient whom the parasites were isolated from had no previous exposure to PMM. A "PMM-R" line was produced from this WT to determine if the selection method had any effect on the fitness of the parasite compared to its WT.

Differences in the susceptibility of PMM-R promastigotes to the effects of SIN and SNAP were observed only in the Sb-I PMM-R clone. Sb-I PMM-R promastigotes were significantly more resistant to the effects of superoxide and NO donation via SIN than Sb-I WT ($p < 0.05$, Figure 6.2 and Table 6.3). When treated with SNAP, there was no observed difference in the susceptibility of promastigotes of Sb-I PMM-R compared to its WT. This suggests that it is the superoxide produced by SIN that Sb-I PMM-R clones have an increased tolerance compared to Sb-I WT. Comparing the effects of SNAP and SIN on promastigotes of Sb-S PMM-R and Sb-R PMM-R to their WTs, no significant difference was noted in either strain with either reactive oxygen species. The effect of treatment with human serum was also measured as a means of testing metacyclogenesis of promastigotes. Again, no significant difference in the rate of metacyclogenesis was detected between any of the three WT isolates and their PMM-R counterparts.

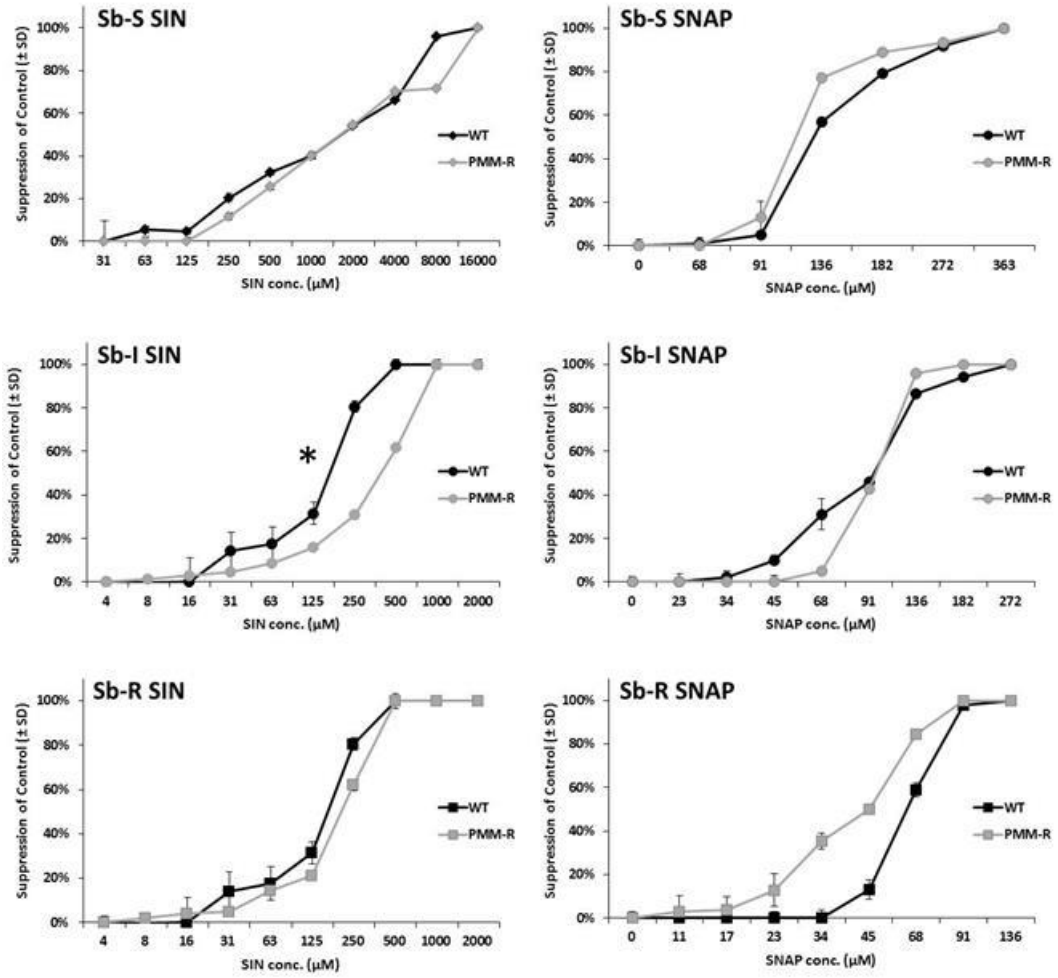


Figure 6.2. The effect of SIN and SNAP on the survival of *L. donovani* promastigotes. Parasite survival was measured using resazurin salt and IC_{50} determined. *p<0.05 for WT vs PMM-R, n=4/treatment.

Intracellular amastigotes were tested for their susceptibility to NO using SNAP and to a combination of the macrophage activating molecules IFN-γ and LPS. No difference was observed between the susceptibility the naturally PMM resistant Sb-S WT isolate and its PMM-R clone that was maintained under constant drug pressure (Table 6.3). Conversely, both Sb-I and Sb-R PMM-R intracellular amastigotes were observed to be significantly more resistant to the effects of SNAP compared to their respective WTs ($p < 0.05$ Table 6.3). While Sb-R PMM-R was 1.5 times more resistant to SNAP that its WT (1283 µM vs 934 µM), Sb-I PMM-R (2456 µM) was almost 3 times more resistant to the effects of SNAP than Sb-I WT (961 µM). Selection for resistance to PMM in all three strains did not appear to impact on the ability of parasites to infect macrophages *in vitro* (Figure 6.3 control columns). Likewise, the effects of IFN-γ and LPS upon intracellular amastigotes appeared to be equally toxic to both WT and PMM-R clones of all three strains (Figure 6.3). Nitrite levels in the supernatant were measured as an indicator for the activation of macrophages. Treatment of amastigotes with IFN-γ and LPS significantly reduced the infectivity and survival of all six WT and PMM-R lines when compared to untreated control infections of macrophages (Figure 6.3). There was however no difference in the infectivity and survival of PMM-R clones compared to their WT when treated with IFN-γ and LPS. Nitrite levels in the supernatant of treated wells were tested to analyse if an infection of intracellular amastigotes was associated with a reduction in macrophage produced nitrite. While nitrite levels increased in correlation with increasing concentration of IFN-γ and LPS, no difference in nitrite production was detected between uninfected, macrophages

and those infected with Sb-S, Sb-I and Sb-R WT and PMM-R clones (data not shown). This indicates that macrophage production of nitrite in response to infection was not hampered by any of the strains.

Table 6.3. IC_{50} values for WT and PMM-R promastigotes and intracellular amastigotes tested *in vitro* to SIN, SNAP and human serum.

	SIN (µM)	**SNAP (µM)**		**Human Serum (µl)**
	Promastigote	**Promastigote**	**Amastigote**	**Promastigote**
Sb-S WT	346 ± 47	117 ± 14	1242 ± 62	3.6 ± 1.1
Sb- S PMM-R	251 ± 39	110 ± 22	1201 ± 103	4.0 ± 1.4
Sb-I WT	239 ± 26	97 ± 5	961 ± 43	2.9 ± 0.9
Sb- I PMM-R	306 ± 29*	96 ± 8	2456 ± 342*	1.8 ±1.9
Sb-R WT	313 ± 29	57 ± 8	934 ± 41	4.3 ± 1.6
Sb- R PMM-R	343 ±37	45 ± 1*	1283 ± 132*	4.7 ± 1.8

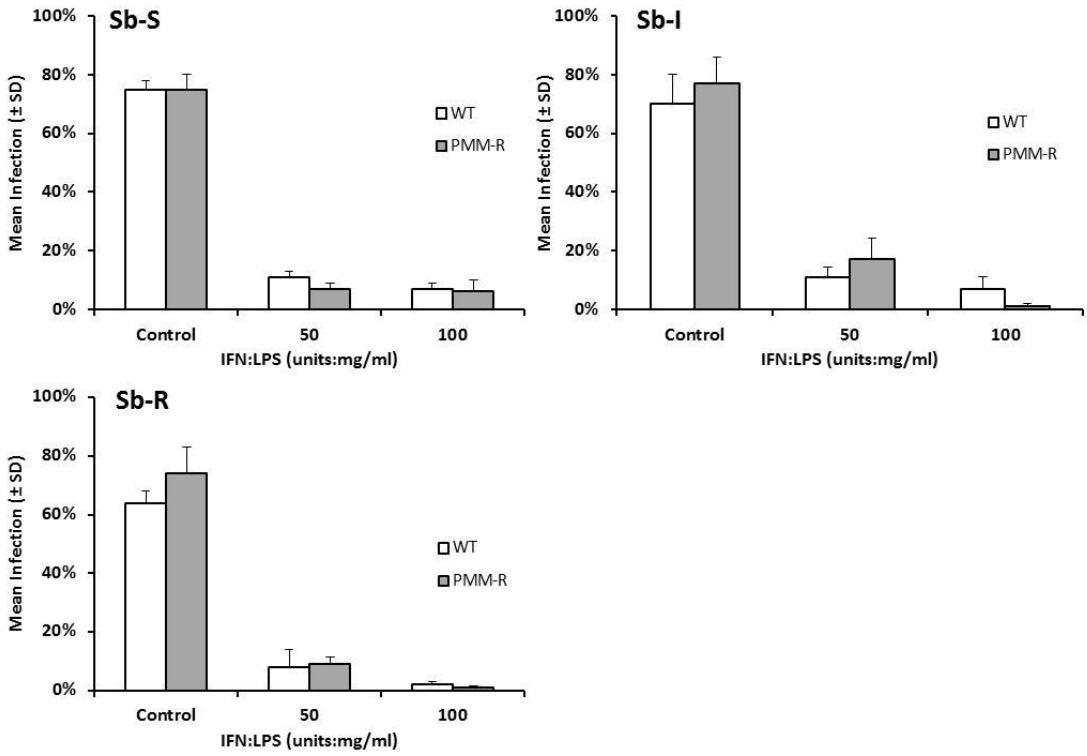


Figure 6.3. Infectivity of WT and PMM-R, *L. donovani* intracellular amastigotes exposed to IFNγ/LPS *in vitro*. Parasite infected macrophages were incubated with IFNγ/LPS for 72 hours and the infectivity of parasites determined by counting Giemsa stained slides.

6.3 Discussion

This study confirms the findings of previous work that demonstrates the capability of *L. donovani* to modulate macrophage immune responses in favour of the parasite's survival (Liu and Uzonna, 2012). It also predicts that selected resistance to MIL and PMM have very different consequences for the fitness of resistant parasites. It is apparent for both drug resistant lines that screening of promastigotes to the effects of SIN and SNAP is not a viable predictor for the amastigote stage as promastigote susceptibility did not correlate with amastigote susceptibility in any of the strains tested.

In general, MIL resistance was associated with a decrease in fitness of both Sb-S MIL-R and Sb-R MIL-R. Promastigote and amastigote stages of both strains appeared to be negatively affected in their susceptibility to superoxide and NO effects with development on MIL resistance. Moreover, the infectivity of Sb-S MIL-R intracellular amastigotes was consistently poor compared to WT. Promastigote cultures were maintained in the laboratory at similar passage numbers in order to account for the decreased infectivity of *Leishmania* with increased culturing. Nevertheless, Sb-S MIL-R promastigotes appeared more impotent than their WT counterpart in their ability to infect macrophages. Intracellular amastigotes did however demonstrate an ability to modulate the host macrophage's production of several cytokines. Both Sb-S MIL-R and Sb-R MIL-R significantly supressed IL-10 and IL-12 compared to uninfected macrophages when stimulated with IFN-γ and LPS. Suppression of nitrite production was a different story however. Sb-S MIL-R and WT amastigote infected macrophages were associated with an increased level of

nitrites at the lower concentration of IFN-γ and LPS. At the higher concentration of stimulant, Sb-S MIL-R nitrite production was significantly lower than that of Sb-S WT but it was comparable to uninfected macrophages, indicating that it wasn't supressing nitrite production at all. Both Sb-R WT and MIL-R appeared to supress nitrite production at the lower level of IFN-γ and LPS stimulation but at the higher level, this effect was lost and in fact Sb-R WT infected macrophages produced significantly more nitrite than those macrophages that were either uninfected or infected with Sb-R MIL-R. This is important to note for the MIL-R parasites in particular as treatment with MIL has previously been shown to prime and increase NO production by macrophages and *Leishmania* infected cells (Zeisig *et al.*, 1995, Ponte *et al.*, 2012). This suggests that tolerance to MIL in our parasites was not achieved via an increased tolerance to cell killing mechanisms induced by MIL treatment. In the previous chapter (Chapter 5), the lipid profile of our MIL-R parasites was illuminated and one important finding was the increase in a number of PC species, particularly in long chain PC observed in Sb-S MIL-R. Long chain fatty acids have previously been shown to have an increased susceptibility to peroxidation (Hulbert, 2005). Here in the Sb-S MIL-R promastigotes we observe an increased susceptibility to the effects of NO in promastigotes. It is possible that this is a direct consequence of the change in lipid composition in the MIL-R parasites. Likewise, the Sb-R MIL-R promastigotes that were observed to have a decrease in the number of long chain PC species compared to WT appear to show an increased tolerance to the effects of SIN in these experiments. We proposed that the loss/mutation in the LdMt gene coupled with the perturbation of the lipid

composition of membranes in out MIL-R parasites is beneficial to the parasite in terms of preventing MIL from entering but with the consequence that it leads to a loss of fitness and a greater susceptibility to macrophage killing effects. This is important as it could imply that while MIL resistance can be easily selected for, it carries with it a major disadvantage to parasite survival in an environment where selective pressure of MIL is not applied. MIL resistance has been selected in laboratory strains numerous times and has always been associated with a disruption of the LdMt gene (Perez-Victoria *et al.*, 2006, Perez-Victoria *et al.*, 2003b, Perez-Victoria *et al.*, 2003a). Despite this, resistance to MIL has not been reported in the field. Relapses have been recorded but drug resistant parasites have yet to be isolated (Rijal *et al.*, 2013). If MIL resistance is associated with a decreased fitness of parasites and general inability to proliferate in macrophages, as observed here, then this could be the reason why. It is interesting to note that all of the studies of MIL resistance, it is the promastigote stages that have been exposed to MIL to generate resistant parasites. Promastigotes are generally used as they are easier to keep in continuous culture and do not require a continuous supply of macrophages. This does however mean that the parasite can develop resistance without having exposure to the effects of macrophage killing. Subjecting intracellular amastigotes to increasing concentrations of MIL would be more labour intensive but it would also help generate resistant parasites that would still have to survive inside their amastigote hosts. It would be interesting and worthwhile to generate MIL-R clones that are derived from amastigotes exposed to MIL to assess their fitness. Resistance to PMM has in fact been performed on amastigotes (Hendrickx *et al.*, 2014). The

study found that not only was PMM-R generated quicker in the intracellular amastigotes but it was associated with a different phenotype than those that were selected for in the promastigote stage. It would also be interesting to analyse the metabolome of MIL-R parasites selected for via the amastigote stage. A lot of importance has been placed on the LdMT gene yet we do not know if it would be such a significant factor if amastigotes were selected for resistance to MIL. After all, it is the amastigote stage that is subjected to MIL when chemotherapy is administered to patients.

The apparent inherent resistance to PMM of Sb-S WT strongly suggests the existence of some isolates in endemic areas of VL that already have a tolerance to the drug in. If this is true then it would have implications for the use of PMM as a treatment for VL in the area. PMM is an attractive choice of chemotherapy because of its relatively low dose cost and administration route although at present it is mainly recommended for use in combination therapy and use on its own is not widespread. If PMM resistance is present in the endemic population then clinical use of the drug could lead to rapid selection of drug resistant parasites and significantly shorten the clinical lifespan of the drug, both in combination or on its own. These data here would suggest that the PMM resistance that was inherent in Sb-S and the selected resistance in Sb-I and Sb-R parasites are stable, even in the absence of exposure to PMM. Importantly too, it appears that PMM resistance is associated with an increased resistance to the cell killing effects of the NO donor SNAP. Both PMM-R clones of Sb-I and Sb-R were significantly more resistant to the effects of SNAP compared to their WTs. Both PMM-R clones of Sb-I and Sb-R were

significantly more resistant to the effects of SNAP compared to their WTs. While only the promastigote stage of Sb-I PMM-R had an increased tolerance to SIN, both intracellular amastigote stages of Sb-I and Sb-R PMM-R had an increased resistance to SNAP compared to their WT. This, as well as the fact that the PMM-R Sb-S WT isolate has a slightly increased tolerance to SNAP compared to Sb-I WT and Sb-R WT shows that there appears, at least in this study, to be correlation between resistance to PMM and an increased fitness against macrophage killing effects. These data would appear to agree with what has already been published on the fitness of PMM-R parasites (Bhandari et al., 2014). Additionally, unlike the MIL-R clones, all our PMM-R clones appeared to infect macrophages at the same rate as their WTs. Another recent study that also used the same Sb-R WT parasites as this study has published similar data in regards to the infectivity of PMM-R parasites (Hendrickx et al., 2015). The most important difference between Hendrickx's work and this is again the fact that our study selected for resistance to PMM in promastigotes while Hendrickx *et al.* selected for resistance in amastigotes. Despite this it appears that both studies agree that PMM-R parasites are no less infective to macrophages and replication in mammalian cells is not hindered with PMM resistance. At first glance it would appear that Hendrickx's data is contradictory to our own as they state there is no fitness advantage to PMM-R parasites. This can however be explained by the fact that their definition of fitness only incorporated study the infectivity of PMM-R parasites *in vitro* and in mice. Their study did not assess the survival of parasites in response to various cell killing mechanisms such as SNAP and SIN which we included in our study. Nevertheless, it appears that both

studies agree on the fact that selection of PMM resistance does not at least confer a negative fitness burden on parasites and they are able to infect and replicate as WT in a variety of tests. If these data can be extrapolated and are true for other PMM-R isolates then it suggests that PMM-R parasites in the wild may have a fitness advantage, even in the absence of PMM. As yet clinical resistance to PMM has not been reported but several studies have shown that it can be easily selected for in the laboratory both in promastigotes and amastigotes (Hendrickx *et al.*, 2014, García-Hernández *et al.*, 2015, Jhingran *et al.*, 2009, Bhandari *et al.*, 2014). Care must therefore be given to the use of PMM and vigilance for the emergence of resistance must occur.

In summary, the main objectives and results achieved in this chapter were

- Resistance to MIL was associated with an overall lack of fitness compared to WT parasites in both promastigotes and intracellular amastigotes in their response to cell killing mechanisms and infectivity.
- MIL-R amastigotes did however maintain their ability to modulate the host macrophage's production of several cytokines.
- In contrast, PMM-R was associated with an increased resistance to the cell killing effects of the NO donor SNAP in both Sb-I and Sb-R PMM-R compared to WT.
- PMM-R parasites appeared to be able to infect and replicate within macrophages at comparable rates to WT.

- The inherently PMM-R Sb-S WT had no discernible change in fitness when continually cultured in PMM (Sb-S PMM-R).

Chapter 7. The Effect of Paromomycin Selection on the Metabolome and Lipidome of *L. donovani*

7.1 Introduction

Paromomycin was first licenced to treat VL infections in 2006 but to date its use has mainly been confined to endemic regions or as part of combination therapy (Sundar *et al.*, 2009). Combination of SSG and PMM is confined to East Africa where, unlike the ISC, treatment with SSG is still viable. Since 2010 in fact, PMM in combination with SSG has been recommended by the WHO as the first line treatment of VL in East Africa (Wasunna *et al.* 2016, WHO Technical Report). With the redundancy of SSG in the ISC, the increasing dependency on MIL therapy and the lack of other affordable options, PMM may become more widely used in the future. It remains the cheapest anti-leishmanial option and although oral MIL is easier to administer, the parental formulation of PMM remains an easily administered drug compared to SSG or AmB. As yet, widespread field resistance has not been reported but naturally resistant PMM-R isolates, such as the Sb-R WT used in this study or those reported by Neal *et al.*, have been isolated from patients (Hendrickx *et al.*, 2014) (Neal *et al.* 1995). In a single dose study, intramuscularly delivered PMM has been shown to peak 1-2 hours after administration at 22 μg/ml and is completely eliminated by 24 hours after treatment (Kanyok *et al.* 1997). With a 15 mg/kg/day dose, the average steady state concentration of PMM in plasma was therefore predicted to be approximately 4.35μg/ml. Similar to MIL, PMM resistance can be easily selected for in the laboratory. These PMM-R strains have alterations in membrane fluidity, decreased PMM uptake and increased expression of ABC transporters such as MDR1, MRPA and protein phosphatase 2A (Bhandari *et al.*, 2014, Jhingran *et al.*, 2009, Maarouf *et al.*, 1998). The involvement of these

transporters indicates that decreased accumulation of PMM inside resistant parasites is at least in part due to an increase in drug efflux from the cell (Bhandari *et al.*, 2014). Although the majority of studies on drug resistance have involved the promastigote stage, a number of recent studies have also generated PMM-R lines using amastigotes, both *in vitro* and *in vivo*. Importantly, generating resistance to PMM in the amastigote stage did not confer resistance in the promastigote stage. Significantly PMM-R amastigotes were only generated when subjected to multiple treatment/relapse cycles in Syrian hamsters (Hendrickx *et al.*, 2014). Similar studies using MIL treatment/relapse selection to produce MIL-R amastigotes were not possible, indicating that treatment failure is perhaps more likely if clinical PMM-R strains are generated. Understanding the mechanisms that are associated with PMM resistance in *L. donovani* could identify strategies to extend the clinical life of this drug if resistance was to emerge.

Very few studies have investigated the effect of PMM treatment or resistance on the metabolome of *L. donovani*. Recently Berg *et al.* completed a study that profiled the effect of selected resistance in *L. donovani* to a range of compounds that included SSG and PMM on their own as well as an SSG/PMM combination (Berg *et al.*, 2015). Both resistant lines had significant alterations in metabolites involved in proline biosynthesis, tryptophan degradation, or the transsulfuration pathway, which have been associated with protection against oxidative stress. Indeed, the PPM-R and PMM/SSG-R strains had an increased capacity for protection against ROS and increased tolerance to H_2O_2 treatment. Furthermore, small changes in the saturation of several glycerophospholipids in resistant lines also correlated with

decreased membrane fluidity. In previous studies (Chapter 6) we identified a strong correlation with resistance to PMM in our selected PMM-R lines and increased fitness in both promastigotes and amastigotes, which included an increased tolerance to oxidative stress. We therefore sought to map the metabolome/lipidome of our three PMM-R clones (SbS, Sb-I and Sb-R PMM-R) compared to their corresponding WTs to identify the mechanism(s) that may be responsible for PMM resistance. Although tandem sequencing of the genome was carried out in PMM-R and WTs, time restraints meant that it was not possible to correlate the alterations in the genome with metabolomic changes. In our studies we used parasites with different inherent Sb susceptibilities to determine if the Sb resistance background impacted on metabolomic changes in PMM-R parasite.

Therefore the main objectives of this study were to:

1) Analyse the metabolomic alterations associated with selected PMM resistance in Sb-S, Sb-I and Sb-R parasites.

2) Compare the metabolic profile of PPM-R and WT parasites to determine if Sb susceptibility impacted on metabolic differences in PMM selected parasites.

7.2 Results

7.2.1. Metabolomic changes associated with PMM-R selection

The entire metabolome of the three *L. donovani* PMM-R clones i.e. Sb-S PPM-R, Sb-I PMM-R and Sb-R PMM-R and their corresponding WTs were extracted and analysed to identify metabolomic changes associated with the PMM resistance. Although Sb-S WT had a natural resistance to PMM, exposing it to PMM selection may identify novel metabolic pathways that are correlated to the continual selection pressure of PMM selection. Therefore, the Sb-S PMM-R line was exposed to the same PPM selection pressure as the PPM-R selected Sb-I and Sb-R lines in order to analyse the effect of the incremental increase in drug pressure, cloning and subsequent constant drug pressure. A total of 265 metabolites were putatively identified in all six types of parasites. Of these, 255 had a mass accuracy of <1 ppm and 15 had a mass accuracy of between 1 and 3 ppm. A PCA plot based on the 265 identified metabolites was generated in order to visualise the major metabolomic differences between each WT and its PMM-R counterpart (Figure 7.1). The first (29.6%) and second (24.3%) principal components clustered Sb-S WT, Sb-S PMM-R and Sb-R PMM-R together. Comparing the PMM-R lines to their WTs, this reflects that Sb-S PMM-R underwent fewer and less pronounced metabolomic changes compared to the Sb-I and Sb-R PMM-R lines. The replicates of each sample were clustered tightly, indicating that variation within each sample was low and results were reproducible.

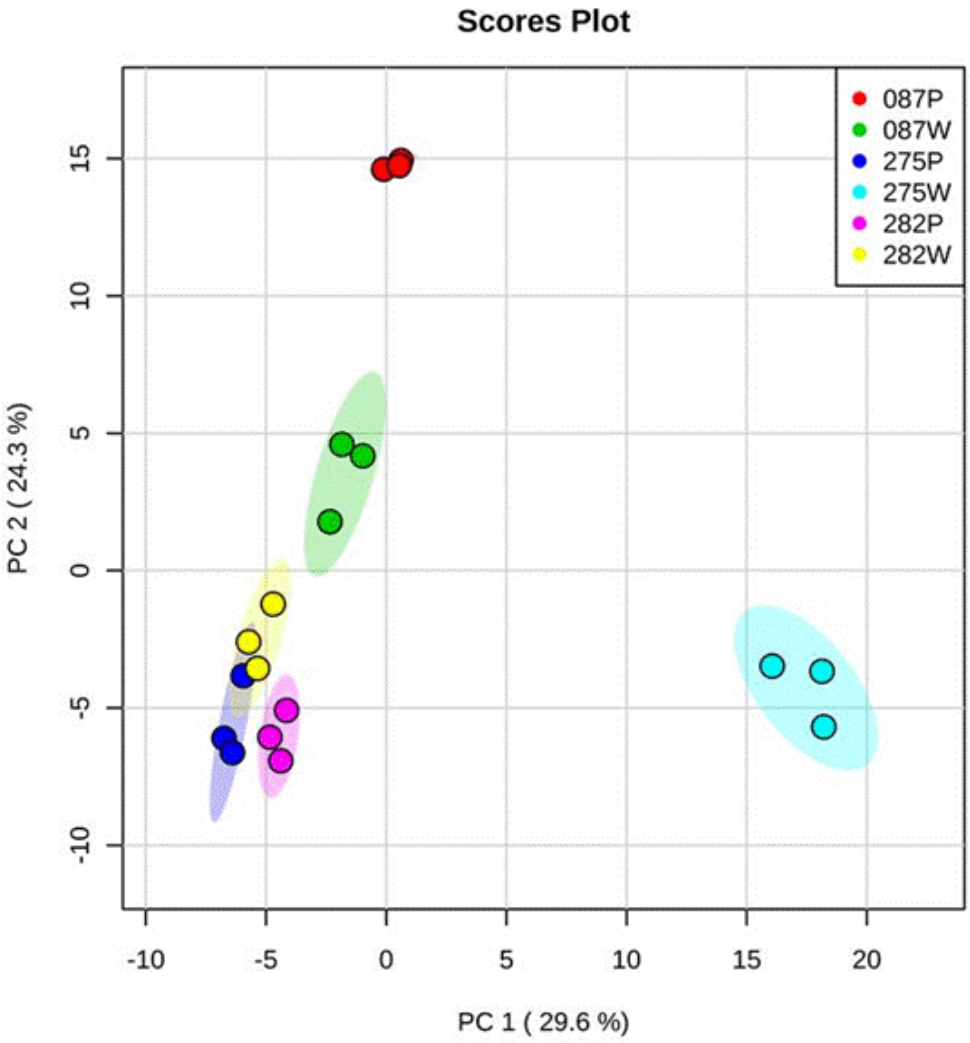


Figure 7.1. Principal Component Analysis (PCA) plot of Sb-S WT (282W, yellow dots), Sb-S PMM-R (282P, pink dots), Sb-I WT (087W, green dots), Sb-I PMM-R (087P, red dots), Sb-R WT (275W, turquoise dots) and Sb-R PMM-R (275P, blue dots). Plot was based on the analysis of 265 metabolites identified in all six lines.

Only 17 metabolites were significantly altered between the Sb-S PMM-R and its corresponding WT, whereas the Sb-I had 22 and Sb-R had 19 significantly altered metabolites compared to their respective WT. There was no common metabolite altered in all three PMM-R clones but 5 metabolites were found altered in at least 2 of the PMM-R lines. Two were common in the Sb-S PPM-R and Sb-I PMM-R (hippurate and hydroxyphenylacetylglutamic acid), two were common to Sb-S PPM-R and Sb-R PPM-R (tryptophan and phenylalanine) and one was common between Sb-I PPM-R and Sb-R PMM-R (proline). The most significantly affected metabolite class of metabolites in all three PMM-R strains was those involved in amino acid metabolism (Table 7.1). In Sb-S PMM-R all 7 metabolites in this class were down regulated compared to its WT, whereas all of these metabolites were up regulated in the Sb-I PMM-R (7 metabolites) and Sb-R PMM-R (9 metabolites) compared to their respective WTs. Proline was found to be upregulated in both Sb-I PMM-R and Sb-R PMM-R along with several of the metabolites involved in proline metabolism. Methionine along with serine and acetyl-homoserine, upstream metabolites of methionine, were additionally found to be up regulated in Sb-R PMM-R. Nine compounds classified as metabolites of carbohydrate metabolism were upregulated in Sb-I PMM-R, indicating that sugar metabolism was significantly increased.

Of all the metabolites altered in response to PMM selection, only tryptophan was found to be significantly different in the WT naturally resistant to PMM (Sb-S)

compared to the other two WTs. After PMM selection, both Sb-S PMM-R and Sb-R PMM-R had significantly lower levels of tryptophan compared to their WTs.

7.2.2 Lipidomic changes associated with PMM-R selection

None of the lipids identified in lipidome studies were significantly altered after PMM-R selection for the Sb-I strain. In contrast, 7 lipids were significantly down regulated and 1 lipid (sphingenine) was up regulated in Sb-R PMM-R compared to its WT. Of these 7, four lipids were small, unsaturated PEs (two diacyl PEs, one alky-acyl PE and one alkenyl-acyl PE). The other three were a ceramide, a PI and an LPE (Table 7.1). In Sb-S PMM-R, there was only a change in one class of lipids compared to WT and this was a significant upregulation in four alkyl-acyl phosphatidylinositols. This may indicate that the upregulation in alkyl-acyl phosphatidylinositols requires parasites to be maintained under PMM pressure.

7.2.3 Genomic changes associated with PMM-R selection in Sb-S WT

As part of the larger Kaladrug project, genome wide sequencing of Sb-S WT and three Sb-S PMM-R clones generated independently from an Sb-S PMM-R parent exposed to 74 μM PMM selection pressure were analysed for difference in their DNA profile and chromosome copy number changes.

Two heterozygous SNPs on chromosomes not associated with copy number changes differentiated Sb-S WT from Sb-S PMM-R samples. One of the SNPs was a nucleotide change at position 651,967 (A1573E, GCA to GAA) in a DNA polymerase zeta catalytic subunit gene (LdBPK_231590). The other SNP observed was a G to A

mutation at site 107, 213 on chromosome 19 situated near a hypothetical gene with unknown function (LdBPK_190280). There were no CNVs or episomes observed that distinguished the three Sb-S PMM-R clones from the Sb-S WT.

Table 7.1. The effect of PMM selection on the metabolome of PMM-R *L. donovani* promastigotes compared to WT. Metabolites that were significantly up regulated are coloured red, down regulated metabolites are coloured blue ($p < 0.05$). Superscript letters in black signify if metabolites part of specific pathways associated with protection against oxidative stress. M = Methionine biosynthesis, P = Proline biosynthesis and TY = Tyrosine biosynthesis. Abbreviations are as follows; HPAGA = Hydroxyphenylacetylglutamic acid, TD = Tetradecanoyl.

	Sb-S PMM-R/WT	Sb-I PMM-R/WT	Sb-R PMMR/WT
Amino Acid Metabolism	Acetyl-serine	Acetyl-lysine	Acetyl-homoserine^{M}
	Glutamatesemialdehyde	Arginine	Carbamoylsarcosine^{P}
	Hippurate	Enol-Phenylpyruvate	Glutamate^{P}
	HPAGA^{TY}	Erythro-Hydroxyglutamate^{P}	Glutamine^{P}
	Phenylalanine	Hippurate	Hydroxyphenyl-lactate
	Tryptophan^{T}	Hydroxyphenyl-propenoate^{TY}	Methionine^{M}
	Tyrosine	HPAGA^{TY}	Serine^{M}
		Methyl-oxopentanoic acid	Phenylalanine
		Proline^{P}	Tryptophan
		Pyrroline-carboxylate^{p}	
		Aspartate	
Carbohydrate Metabolism		Acetolactate	Coenzyme A
		Arabinonate	Xylonolactone
		Diacetyl	
		GDP-mannose	
		Glycogen	
		Myo-Inositol	
		Ribose-phosphate	
		Trehalose-phosphate	
		Trihydroxy-butanoic acid	
Lipids	PI(O-16:0/18:2)		Sphingenine
	PI(O-16:0/20:2)		Cer(d18:0/17:0)
	PI(O-18:0/17:2)		LysoPE(0:0/18:2)
	PI(O-16:0/19:1)		PE (16:0)
	Eicosanoyl-sphingenine		PE (18:1/18:1)
	Hexadecasphinganine		PE(O-20:0/17:2)
	Palmitoyl methionine		PE(P-18:0/17:2)
	Sphinganine		PI(O-16:0/20:1)
Others	Beta-aspartyl-threonine	Sinapyl alcohol	
	Dodecanoic acid	Heptaprenyl-hydroxybenzoate	
		Octaprenyl-hydroxybenzoate	

A dose dependent aneuploidy signature was associated with PMM selection for Sb-S PMM-R. Seven chromosomes (6, 8, 13, 14, 15, 22 and 32) were increased from disomy to trisomy in all three PMM-R clones (Figure 7.2). And a further disomy to trisomy transition was also found for chromosome 7 in two of the three Sb-S PMM-R clones. No other change in chromosome copy number was consistently observed for all three Sb-S PMM-R clones.

The effect of PMM selection on chromosome copy number was determined for un-cloned intermediate lines selected after exposure to 2, 4 and 8 µM PMM. At concentrations of 2 and 4 µM PMM, there was no consistent alteration in chromosome copy number or SNP change in selected parasites and their respective WT. However, at 8 µM PMM, chromosomes 6, 8, 13, 14, 15, 22 and 32 all had mean chromosome copy numbers between 1.16 and 1.33. This indicates that the PMM selected parasites contained a mixed population of disomic and trisomic parasites. Likewise, the SNPs A1573E and one on chr19 had frequencies of 0.16 and 0.74 respectively, which reflects the above chromosome copy number shift. These changes increased in frequency in the independently derived Sb-S PMM-R parents, where the copy number for all 7 chromosomes was trisomic, and the SNP A1573E had a frequency of 0.31 and the SNP on chr19 was fixed in all of the PMM-R parents.

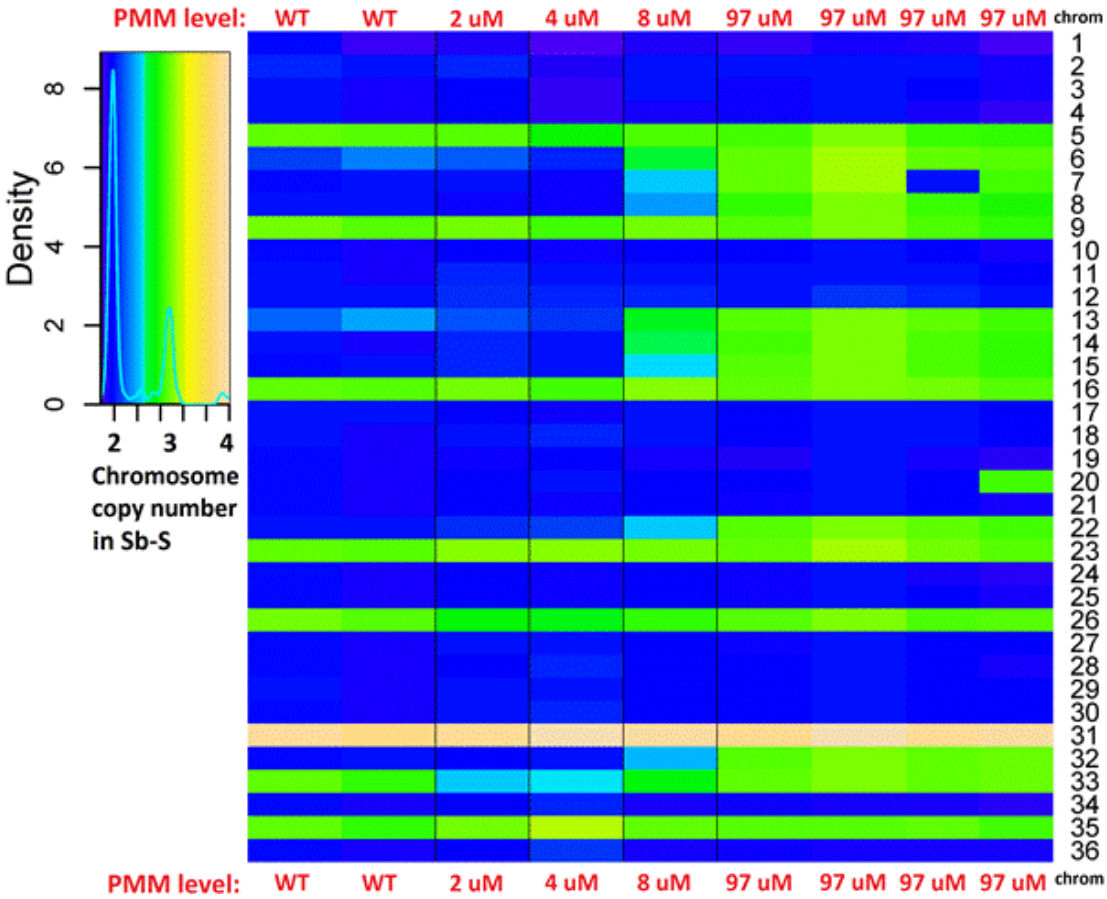


Figure 7.2. The effect of PMM selection on chromosome copy number for Sb-S *L. donovani* promastigotes. Sb-S WT was selected for resistance to 8 µM PMM and this parasite was then used to produce three independent PMM-R parents. DNA isolated from one PMM-R clone/ PMM-R parent was used in studies to determine the number of copies present for each chromosome. Figure produced by Dr. Tim Downing and used with permission.

5.3 Discussion

The influence of PMM resistance on the metabolome, lipidome and genome of different *L. donovani* isolates was determined by exposing promastigotes to increasing of concentration of PMM. Sb-S WT was inherently resistant to PMM; however, comparisons between Sb-S WT and Sb-S PMM-R could identify PMM specific resistance mechanisms or identify changes that are only expressed under PMM selection pressure.

Resistance to PMM in Sb-I PMM-R and Sb-R PMM-R was associated with significant increases of the amino acids proline, methionine and their derivatives compared to their WTs, which are all known to have a role in protection to oxidative stress. This correlates with the significantly higher tolerance to SNAP (Sb-I PMM-R) or SIN (Sb-R PMM-R) for PMM-R parasites compared to their WT counterparts also found during the course of this study (Chapter 6). Proline is known to be a scavenger of free radicals and therefore increased levels would protect the parasite against these radicals produced as part of the host's protective immune responses (Kaul *et al.*, 2008). Increasing proline levels in resistant strains may be fundamental in mediating protection against abiotic stress as it is present in plants, bacteria, parasites and cancer cells (Magdaleno *et al.*, 2009, Natarajan and Becker, 2012, Qamar *et al.*, 2015). In *T. cruzi*, supplementation of proline has been shown to increase parasite resistance to ROS and the drugs nifurtimox or benzidazole *in vitro* (Sayé *et al.*, 2014). Moreover, complemented expression of proline dehydrogenase, the enzyme that catalyses proline conversion to pyrroline-5-carboxylate, in *Saccharomyces cerevisiae* was demonstrated to reduce intracellular levels of proline and increase

sensitivity to oxidative stress in comparison to null mutants (Paes *et al.*, 2013). Previous studies have shown that *L. donovani* parasites exposed to osmotic stress and nutrient starvation have increased levels of proline in response to these stresses (Inbar *et al.*, 2013, Martin *et al.*, 2014). Recently, Berg *et al.* 2015 reported, amongst other metabolomic differences, an upregulation in proline in the *L. donovani* strain MHOM/ET/67/HU3 made resistant to PMM or a combination of PMM/SSG (Berg *et al.*, 2015). In the same study they found that supplementing promastigotes with 200 mM proline increased their Sb^{III} tolerance 2 fold compared to un-supplemented parasites. In contrast, supplementation did not affect the tolerance of parasites towards AmB or MIL, indicating that supplementation with proline gave benefits that were drug dependent. It is interesting that the Sb susceptibility of WT strain did not influence the proline levels of the corresponding PMM-R strain in Sb-I or Sb-R WTs compared to the Sb-S WT. Sb^{III} is known to induce oxidative stress and a resistance to the compound has been correlated with a greater tolerance to oxidative stress therefore an increase in proline could have been reasonably postulated in those parasites that had an increased tolerance to Sb^{III} or Sb^{V}. The observed disparity in proline levels between Sb-S WT and the other WTs may reflect the inherent resistance of the Sb-S WT to PMM and the inherent higher proline levels in-line with their Sb resistance. Ideally the metabolome of more Sb-S WT isolates with no inherent resistance to PMM should be analysed to elucidate if there is indeed a correlation between intrinsic proline levels and resistance to both SSG and PMM. Intracellular levels of proline therefore appear to provide a marked benefit in resistance to oxidative stress in the eukaryotic cell.

Therefore it is perhaps not surprising that proline and one of its precursors, pyrroline-5-carboxylate, were significantly upregulated in Sb-I PMM-R. Although Sb-R PMM-R did not demonstrate an increase in proline or pyrroline-5-carboxylate, other metabolites that are associated with resistance to oxidative stress were found to be up regulated after PMM selection. Significantly higher amounts of methionine and two downstream metabolites, serine and acetyl-homoserine were found in Sb-R PMM-R. Methionine has been associated with increased tolerance to oxidative stress and methionine sulfoxide, the product of oxidised methionine, has also been shown to protect *L. major* from the effects of ROS (Sansom *et al.*, 2013). Together, these observations suggest that PMM selection can induce independent metabolic changes yet produce a common phenotype, namely an increased resistance to PMM by way of increased oxidative stress tolerance.

Sb-I PMM-R had significantly higher amounts of 9 metabolites involved in carbohydrate metabolism compared to its WT, indicating that increased glycolysis may be related to PMM resistance. The exact nature of this upregulation is unclear as none of the metabolites directly link with one another. A recent study showed that *L. donovani* parasites exposed to oxidants *in vitro* or *ex vivo* significantly rewired their glucose metabolism from glycolysis towards a pentose phosphate pathway. In the study, glucose-6-phosphate dehydrogenase and transaldolase were both significantly upregulated in response to lethal concentrations of ROS (Ghosh *et al.*, 2015). Although neither of these particular metabolites were significantly altered in Sb-I PMM-R, eight metabolites involved in carbohydrate metabolism were

significantly upregulated, indicating a significant reconfiguration of glucose metabolism in response to PMM selection.

The influence of PMM pressure on the Sb-S WT, which was inherently resistant to PMM compared to the other WTs, was investigated by selecting for resistance to 97 µM PMM. Seven amino acids were significantly downregulated in Sb-S PMM-R after PMM selection. Of these, only two, tyrosine and hydroxyphenylacetylglutamic acid, are connected in the same metabolic pathway (tyrosine metabolism). The phosphorylation of this amino acid by tyrosine phosphatase is important in cell signalling and has been correlated with increased virulence in *Leishmania spp.* (Gomez *et al.*, 2009). Interestingly the four phosphatidylinositols (PIs) upregulated in Sb-S PMM are also known to have roles in cell signalling in *L. donovani* (Zhang and Beverley, 2010).

Overall the metabolic studies indicate that the PMM-R strains have common and strain-specific features. Sb-R PMM-R parasites additionally had a downregulation of 7 lipids, of which 4 were PEs and one an LPE. The PCs and PEs content of *L. donovani can* have an impact on membrane fluidity, with a decrease in PE content associated with an increase in the fluidity of the cell membrane. Increased membrane fluidity associated with PMM resistance has previously been reported by Bhandari who used clinical isolates from India (Bhandari *et al.*, 2014). However studies by Berg have indicated that PMM selection was associated with a decrease in membrane fluidity using parasites isolated from Nepalese patients (Berg *et al.*, 2015). The Sb-I

PMM-R had no difference in its lipid content compared to its WT, indicating that changes in membrane fluidity are not essential for PMM resistance.

There was a significant upregulation in four alkyl-acyl phosphatidylinositols in the In Sb-S PMM-R compared to its WT but not the other PMM-R parasites. This may indicate a lipid change that only occurs if an inherently PMM resistant parasite is maintained under drug pressure. Phosphatidylinositols are minor components of plasma membranes and also serve as precursors for GPIs. In eukaryotes GPIs are important signal transducer and protein anchoring glycolipids that attach post translationally to the cell surface (Perino *et al.*, 2012). Changes in the PI content could therefore affect the structure or presence of GPIs on the surface of PMM-R resistant parasites, which could indirectly affect PMM uptake or export.

Only one metabolite, tryptophan, was significantly altered with PMM selection and found to be altered in Sb-S and Sb-R WT parasites (Chapter 4). Tryptophan was found in significantly higher levels in Sb-R WT compared with the inherently PMM resistant parasite Sb-S WT. Paromomycin selection on both Sb-S and Sb-R also correlated with a reduction in tryptophan, suggesting that reduced levels of tryptophan maybe a consequence of adaptation to PMM or beneficial in resistance to the drug.

Analysis of the genetic changes associated with PMM selection in the Sb-S WT strain indicated that aneuploidy was the predominant genetic response to PMM pressure in all three lines analysed. Despite duplication in 7 chromosomes, there were surprisingly few metabolites or lipids upregulated in Sb-S PMM-R clones. This could

however simply reflect that the previous genetic changes are sufficient to confer PMM resistance. Given that Sb-S WT was inherently resistant to PMM and the selection of PMM-R parasites was more akin to continual drug pressure rather than an increase in resistance, the small number of changes in the metabolome is not particularly surprising. Instead, the switch from disomy to trisomy in 7 chromosomes was more unexpected. As discussed in the previous chapter (Chapter 5) aneuploidy would appear to be an early response of *Leishmania* when exposed to environmental stress such as drug pressure. Although Sb-S WT was inherently resistant to PMM, those parasites in the mixed population that have increased their chromosome numbers obviously have an advantage which resulted in the three different PMM-R clones of Sb-S showing aneuploidy.

Selection at 2 and 4 µM PMM did not appear to have an effect on the genome of Sb-S exposed to the drug. It therefore seems that 8 µM PMM is sufficient to elicit a stress response as evidenced by the consistent increase in dosage across seven chromosomes. PMM tolerance in Sb-S was also linked to two SNPs, one of which is likely to alter protein function.

The A1573E change at position 651,967 is situated in a DNA polymerase zeta catalytic subunit gene (LdBPK_231590). This enzyme is involved in DNA replication (EC 2.7.7.7) and the product of this gene is expressed throughout the life cycle (Leifso *et al.*, 2007). The change from a hydrophobic alanine to a polar, negatively charged glutamate has would have functional impacts on protein function (Adzhubei *et al.*, 2010). The exact nature of this effect and whether it causes an increase or decrease in protein activity is however not clear.

In summary, PMM-R parasites were compared to their WT counterparts to establish a correlation between selection of drug resistance and changes in the metabolome. The main outcomes identified were:

- PMM selection was strongly correlated with an increase in metabolites involved in both amino acid and glucose metabolism. Specifically, parasites resistant to PMM had significantly increased levels of metabolites correlated to protection against oxidative stress, particularly proline, methionine and their associated metabolites.
- The naturally PMM-R, Sb-S WT was associated with relatively few metabolomic changes when kept under continual drug pressure, indicating that PMM resistance was stable.
- Seven chromosomes were increased from disomy to trisomy and two SNPs were common to all three Sb-S PMM-R clones sequenced.

Chapter 8. Novel Minor Groove Binders for the treatment of *L. donovani*

8.1 Introduction

Novel chemotherapeutic agents to treat VL are required due to treatment failures of current VL chemotherapy, which may be partly caused by increased drug resistance in endemic parasite populations. Unfortunately, the drug discovery pipeline for chemotherapy specifically designed to treat leishmaniasis is limited. A small number of compounds are currently undergoing research, Drugs for Neglected Diseases initiative (DNDi), a non-profit, collaborative research and development organisation is leading the development of several compounds with the potential to treat VL patients (www.dndi.org). The Hit to Lead program is designed to identify and optimise compounds with potency against *Leishmania spp.* identified from high throughput screens. One promising set of compounds identified from this program is the amino-pyrazole series (Mowbray *et al.* 2015). Several of these compounds have been shown to have high potency *in vitro* with IC_{50}s of 2.4 µM and 1.3 µM against *L. infantum and L. donovani* respectively as well as stability and activity in hamster models. Further down the pipeline, fexinidazole in combination with MIL for treatment of patients in East Africa is also being developed by DNDi. In parallel, it is undergoing phase II clinical trials against VL and clinical trials against stage 2 human African trypanosomiasis (Nagle *et al.* 2014). The small Phase II proof-of-concept study in Sudan saw 3 out of 14 patients cured of VL after 6 months but the trial was halted as efficacy was not proven. According to the website, DNDi are planning a study to assess the pharmacokinetics and safety of administering both MIL and fexinidazole together (www.dndi.org). Piggy backing on broad spectrum drugs or repurposing of drugs designed for other indications, are

attractive options. Such tactics have proved useful in the past to treat VL and other parasitic diseases (Nagle et al., 2014). For example, the anti-parasitic properties of MIL were discovered in the UK at the same time as the drug was being assessed as an anti-cancer treatment in Germany (Croft & Engel 2006). PMM is a broad spectrum antibiotic and AmB is only financially accessible to VL patients in the ISC because it is manufactured as an anti-fungal agent and made available to VL patients by a preferential price agreement negotiated by WHO (Sundar and Chakravarty, 2010). Other neglected tropical diseases have also benefited from repurposing and piggybacking on chemotherapy such as eflornithine, originally developed in the 1970s as an anti-cancer treatment (Steverding, 2010). Specifically designing a drug to treat VL does not make an attractive business proposition as no matter the cost of research and development, profitability is compromised by the poverty that afflicts those at risk of the disease (Nagle et al., 2014). Novel compounds are more likely to be designed within the academic sector where focus is less profit driven. With these facts in mind, we began testing a class of minor groove binding (MGB) compounds synthesised in the chemistry department at Strathclyde, which have broad spectrum activity against various microorganisms, for activity against *L. donovani*.

As part of the helical structure of DNA, sugar phosphate backbones form two distinct sizes of grooves classified as a major groove of 22 Å and a minor groove of 12 Å in diameter (Figure 8.1). Proteins are capable of binding to either of the grooves but small molecules (<1000 Da) have a propensity for binding to the minor groove. These compounds are a large class of molecules that are characterised by

their affinity to bind to the minor grooves of DNA, thus disrupting its normal function and replication. They have been shown to possess a broad spectrum of anti-bacterial and anti-parasitic activity (Barrett et al., 2013, Khalaf et al., 2011, Parkinson et al., 2013, Khalaf et al., 2012a). Binding of MGBs to DNA is known to occur with a high affinity to adenine-thymine (A-T) rich sequences of the minor groove. The mode of action of MGBs is varied, with some such as CC-1065 that cause permanent damage to DNA via cleavage of the backbone, and others such as pentamidine that reversibly inhibit DNA-dependent functions by forming non-covalent complexes with DNA (Baraldi et al., 2004).

One class of MGBs known as diarylamidines were shown to be effective against leishmaniasis and trypanosomiasis as far back as the 1930s. Several diarylamidines, including DAPI and pentamidine, were synthesised in the 1930s in an effort to develop chemotherapy against these protozoa (Baraldi et al., 2004).

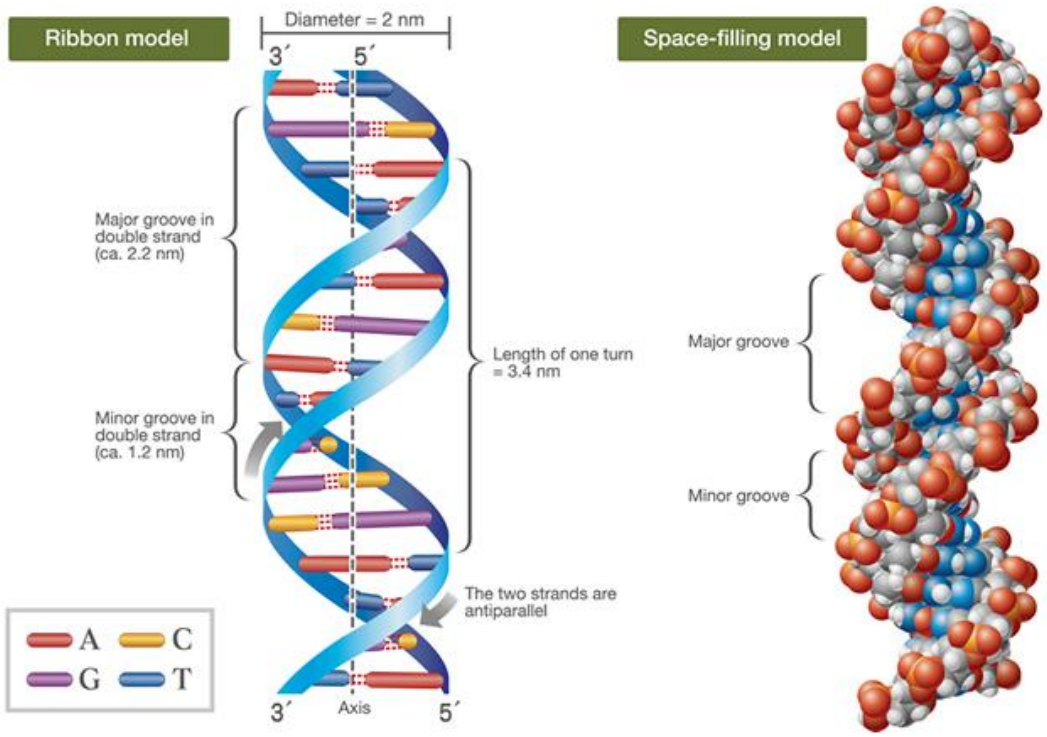


Figure 8.1. Diagrammatic representation of the major and minor grooves formed in the helix of a strand of DNA. Modified from http://www.bio.miami.edu/tom/courses/bil255/.

Although DAPI was associated with a number of undesirable side effects eventually abandoned as a drug, its natural fluorescence and affinity to bind to DNA has led it to be used routinely as a differential stain in fluorescence microscopy. Pentamidine, an aromatic diamidine, is still used today to treat *Trypansoma brucei gambiense*, *Pneumocystis jiroveci* and can also be used to treat *L. donovani* (Baraldi et al., 2004). Several naturally occurring MGBs, such as the structurally similar netropsin and distamycin A, have been isolated from *Streptomyces spp.* and are active against viruses, bacteria and protozoa. Netropsin is a dipyrrole whereas distamycin A has an additional pyrrole group. Both compounds have activity against a number of micro-organisms but they lack specificity and also bind to mammalian DNA, causing unwanted side effects (Olsen et al., 2003, Chen et al., 1994). Several analogues of these compounds have been synthesised in recent years with the aim of reducing toxicity and improving specificity (Khalaf et al., 2004, Khalaf et al., 2012b). Recently, the Suckling group at the University of Strathclyde has synthesised three lead MGB compounds (MGB-1, 2 and 3) as well as a number of derivatives based around the structure of Distamycin A. In particular, MGB-3 has been shown to have significant activity against gram positive bacteria with no reported toxicity against mammalian cells. This compound has been commercialised by MGB Biopharma and is currently undergoing clinical trials against *C. difficile* (unpublished data). Previous studies have shown that subtle structural alterations to tail groups or increasing alkyl chains on the parent compounds can have dramatic effects on the biological activity of these compounds (Khalaf et al., 2012a, Khalaf et al., 2011). For this reason, the activity of the three lead MGB compounds as well as a set of derivatives with

functional group alterations were tested against *L. donovani*. The MGBs screened in this study were variants of the 3 lead MGB compounds including variations in; their n-terminus headgroups, c-terminus tails, alkyl sidechain alterations or the addition of n-oxide or methyl groups. Within this compound library, several MGBs have previously been reported to have activity against a variety of microorganisms including *Trypanosoma brucei brucei, Aspergillus niger* and *Candida albicans* and we sought to investigate their activity against *Leishmania* (Anthony *et al.* 2007. Scott *et al.* 2015).

Compounds were tested *in vitro* using macrophages infected with luciferase expressing *Leishmania* promastigotes as this allowed higher throughput screening against the clinically relevant amastigote stage compared to the traditional methods using Geimsa stained cells. Cells were infected for 24 hours to allow the parasites to enter macrophages and begin transformation into the amastigote stage. The cells were drug treated and then parasite levels were determined 72 hours post-infection by assessing the amount of bioluminescence (BLI) emitted by cells treated with luciferin solution. BLI is produced as a by-product of an enzymatic, oxygen and energy dependent reaction of luciferase on the substrate luciferin (Andreu *et al.*, 2011). Parasites expressing the luciferin substrate, luciferase, can be used for both *in vitro* and *in vivo* experiments to monitor parasite growth and infection (Claes *et al.*, 2009). *Leishmania* parasites expressing the luciferase reporter gene have been widely used *in vitro* to screen drugs and identify potent compounds (Lang *et al.*, 2005, Ravinder *et al.*, 2012, Voak *et al.*, 2014). Additionally, we have previously used luciferase expressing parasites to monitor disease progression and as a model of

drug delivery *in vivo* (Alsaadi *et al.,* 2012). In particular, luciferase expressing parasites are useful in non-invasively monitoring the course of infection in animal models. Traditional methods for monitoring disease progression of leishmaniasis in mice rely upon estimating parasite loads in the liver, spleen and lymph nodes. This requires microscope examination or molecular techniques to quantify parasite DNA however the major drawback is that these techniques require the infected animal to be sacrificed and so can be both costly and unnecessarily wasteful. Luciferase expressing parasites negate this need to sacrifice individual animals to take liver and spleen smears and instead allow continual monitoring of infections *in vivo*. This not only significantly reduces the number of animals that need to be killed but also reduces inter-animal variation and a reduction in errors and so can also improve precision and reliability of data (Baker, 2010). Firefly luciferase protein is widely used as it is highly sensitive, has a low background luminescence in animals and has quantitative correlation between signal strength and cell number (Claes *et al.*, 2009). A gene encoding luciferase protein, from fireflies (*Photinus pyralis*), was integrated into the genome of *L. donovani*. Integrating the luciferase gene into ribosomal DNA allowed the luciferase protein to be constantly expressed by the parasite, meaning that luciferase expression did not need to be turned on. In order to measure bioluminesce, the luciferase enzyme produced by the parasites must come in contact with its substrate, luciferin, in the presence of ATP. This reaction creates the products oxyluciferin, adenosine monophosphate and a photon of light. It is this photon of light that is used as the measurable product when monitoring luciferase parasites using bioluminescence (Brogan *et al.*, 2012).

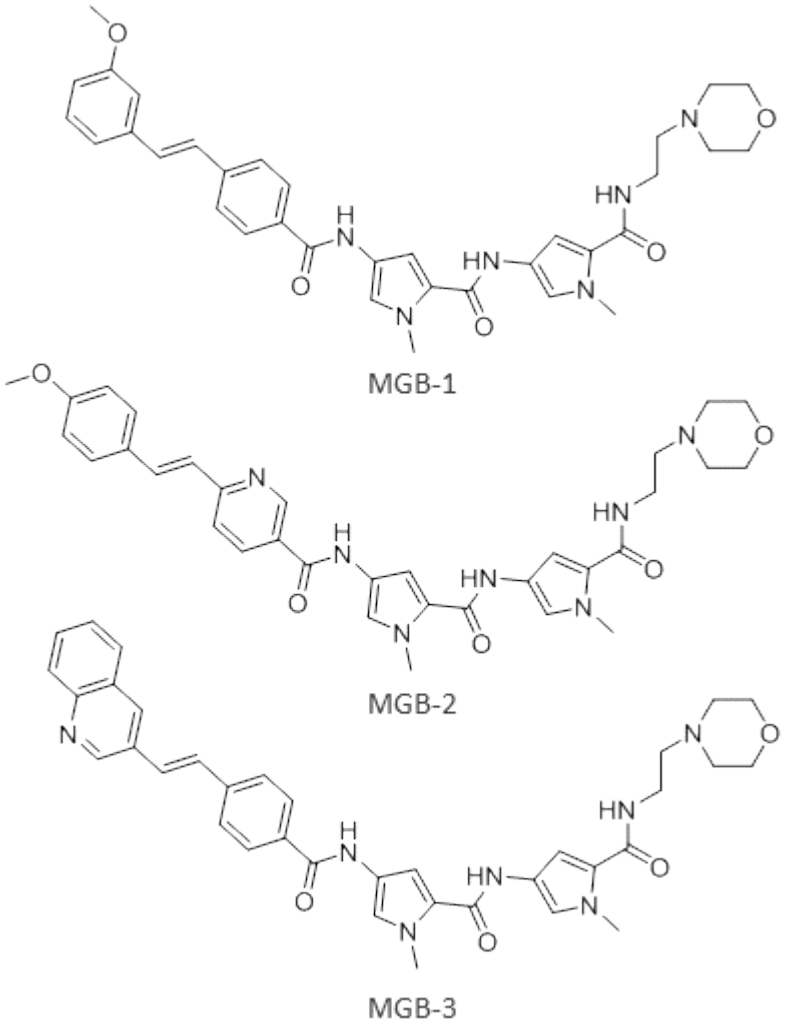


Figure 8. 2. Structure of the three lead compounds tested; MgB-1, MGB-2 and MGB-

3.8.2 Results

8.2.1 Validation of luciferase expressing *L. donovani*

The correlation between BLI produced by luciferase promastigotes and the number of parasites was assessed to determine if BLI production was linear and reliable (Figure 8.2). The correlation coefficient of the linear fit of 0.99 indicated that there was a good correlation between the light emitted and the number of parasites. This parameter was routinely checked in experiments and longitudinal studies showed the amount of BLI produced /parasite was stable over time.

The *in vitro* susceptibility of intracellular *L. donovani* Luc parasites to standard anti-leishmanial drugs was tested to validate the method used and determine if integration of luciferase gene had altered the susceptibility to standard drugs, thus compromising their suitability for screening. Luc parasite susceptibility was calculated using the luminescence method and confirmed with counting of Giemsa stained macrophages under the microscope. WT parasite IC_{50}s were determined purely by counting Giemsa stained infected macrophages. Both WT and their luciferase-expressing counterparts had similar susceptibilities to SSG, AmB, MIL and PMM (Table 8.2) so confirmed the susceptibility of this parasite strain to the commonly used anti-leishmanial drugs. This validated that Luc parasites had similar susceptibility to anti- leishmanial compounds as WT and would therefore be suitable as a high throughput screening model to test novel compounds.

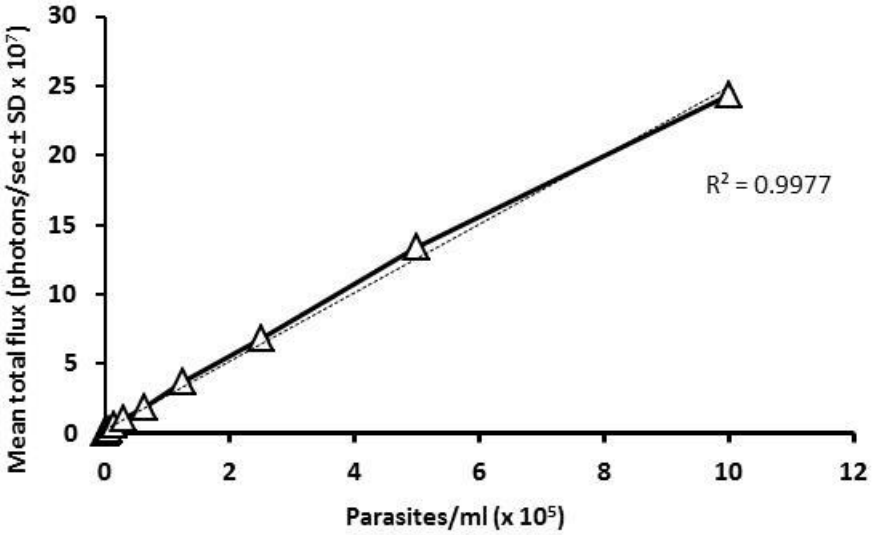


Figure 8.3. Correlation between BLI and parasite number for of *L. donovani* Luc 1.2 promastigotes measured using the IVIS system. The light emitted starting with $1x10^4$ parasite /ml followed by double dilution, determined after incubated of cell in 150 µg/ml luciferin solution. n = 6/treatment.

Table 8.1. The effect of insertion of the luciferase gene on the *in vitro* susceptibility of *L. donovani* to standard anti-leishmanial drugs. IC_{50}s were calculated using BLI (luc 1.2) or direct counting of intracellular amastigotes (WT) using bone marrow derived macrophages from BALB/c mice. Values are the mean of 3 experiments (± SD), n = 3-6/treatment.

	Mean IC_{50} (± SD)			
	SSG (mM)	AmB (μM)	MIL (μM)	PMM (μM)
WT	2.1 ± 1.0	0.1 ± 0.0	0.8 ± 0.4	0.7 ± 0.6
Luc 1.2	2.6 ± 0.3	0.1 ± 0.0	0.7 ± 0.1	0.7 ± 0.1

8.2.2 The effect of MGBs on the *in vitro* survival of *L. donovani*

An initial *in* vitro screen of 29 MGBs against *L. donovani* amastigotes infecting macrophages was undertaken to determine if there was any structural relationship to the antileishmanial activity. Incubation with MGBs at a concentration of 12.5 μM caused a significant suppression in parasite numbers in infected macrophages (Figure 8.3) and there was a difference in activity between compounds. All but two of the compounds tested had an alkene headgroup. The three exceptions were FS-4/69, T/28 and T/29 that contained an amide, amine and amine headgroup respectively. Of these, the amide FS-4/69 had an activity below 50% while the two amine headgroup compounds showed a complete lack of activity against *L. donovani*. Interestingly, the methylated morpholine (-ME) and n-oxide morpholine (-NO) tail derivatives of the three lead MGB (-1, 2 and 3) all had higher activities than their parent compounds. Similarly, several of the alkyl morpholine tail derivatives (A-prefix) also showed a greater activity than the original MGBs. Only one of the truncated MGB compounds (T-prefix), T4/50 showed extremely high

activity while five other truncated MGBs were inactive (Figure 8.3 and Table 8.2). Importantly, three of these truncated compounds that showed no activity had a piperazine tail group (Table 3). The lipophilicity of the MGBs was predicted by a log $D_{7.4}$ value (Table 8.3). There did not appear to be a correlation between the lipophilicity of MGBs and their activity against *L. donovani in vitro* (data not shown).

The activity of fifteen of the most active MGBs were analysed in further detail and the IC_{50} values of the compounds against L*. donovani* were ascertained (Table 8.2). IC_{50} values ranged from 1.0 – 5.7 µM and as suggested from the initial screens, the N-oxide or methylation variants of the lead MGBs were most active against *L. donovani* (Figure 8.3). MGB-3-NO was the most active compound with an IC_{50} of 1.0 µM (Table 8.2 and Figure 8.4A). In comparison to current anti-leishmanial drugs, several of the compounds were found to have IC_{50}s that were comparable to that of MIL and PMM however AmB was significantly more active than all of the MGBs tested (Table 8.1 and 8.2).

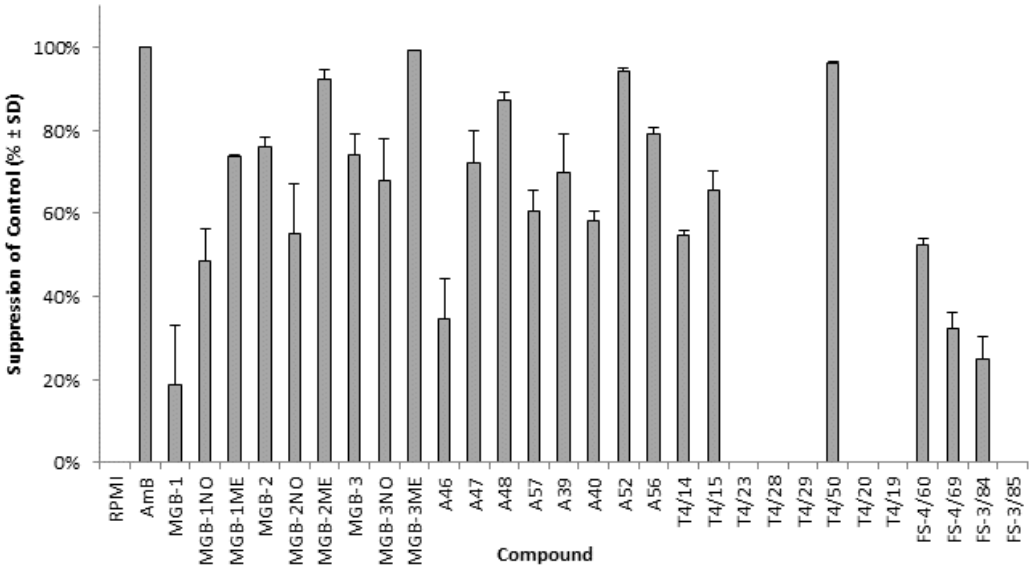


Figure 8.4. The effect of treatment with different MGB compounds on the *in vitro* survival of *L. donovani* Luc 1.2 in infected macrophages. Values are representative of one of two separate experiments, where n = 3/drug treatment and n-=6 for controls (RPMI). NO suffix = n-oxide morpholine derivative, ME suffix = methylated morpholine derivative, A prefix = morpholine side chain derivative, T prefix = truncated and FS prefix denotes unclassified derivative.

Table 8.2. IC_{50} values for selected MGB compounds against *L. donovani* Luc 1.2 *in* infected macrophages. Values are the mean of 3 experiments (± SD), n = 3/treatment.

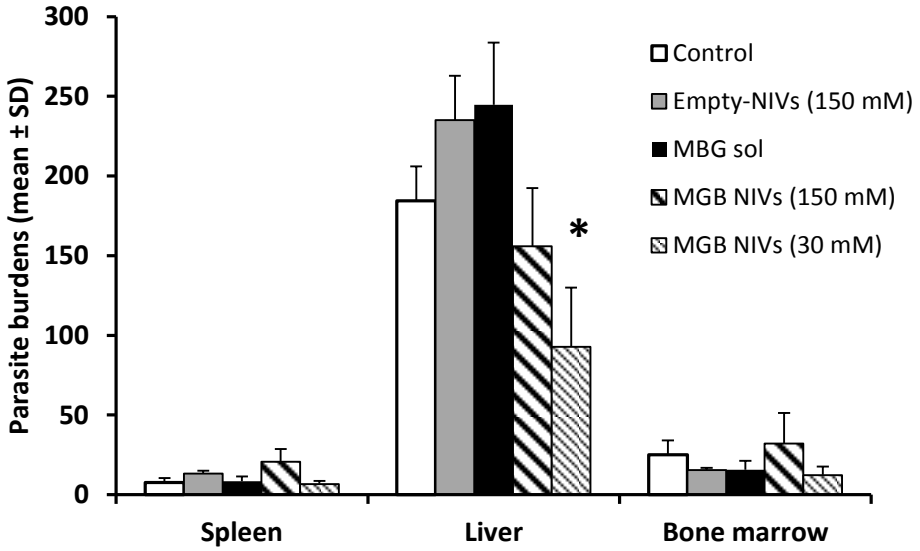
Compound	**Mean IC_{50} ± SD (µM)**	**Mean IC_{90} ± SD (µM)**
AmB	0.1 ± 0.0	0.7 ± 0.2
MIL	0.7 ± 0.1	2.2 ± 0.6
PMM	0.7 ± 0.0	4.8 ± 1.7
MGB-1	3.5 ± 0.1	10.9 ± 0.1
MGB-1-NO	2.4 ± 0.3	10.6 ± 0.4
MGB-1-ME	2.5 ± 1.1	4.9 ± 0.4
MGB-2	5.7 ± 0.4	10.4 ± 0.7
MGB-2-NO	3.1 ± 1.0	9.0 ± 1.8
MGB-2-ME	1.4 ± 0.7	4.6 ± 0.1
MGB-3	5.1 ± 0.7	13.9 ± 0.6
MGB-3-NO	1.0 ± 0.7	2.7 ± 0.1
MGB-3-ME	3.6 ± 0.4	9.7 ± 0.9
A39	3.1 ± 0.2	6.9 ± 0.6
A47	5.2 ± 0.7	9.9 ± 2.3
A48	4.1 ± 1.0	7.8 ± 1.7
A52	3.8 ± 0.5	9.5 ± 0.8
A56	5.1 ± 0.2	11.5 ± 2.2
T4-50	1.6 ± 0.9	5.4 ± 0.7

Table 8.3. MGB compounds and a summary of their head- and tail-group structures and estimated lipophilicitymeasured by Log $D_{7.4}$.

MGB	Mw	Head Group	Tail Group	Log D
A39	867.79	Alkene	Morpholine	3.51
A40	881.82	Alkene	Morpholine	4.04
A46	887.82	Alkene	Morpholine	4.81
A47	901.85	Alkene	morpholine	5.34
A48	887.82	Alkene	Morpholine	4.81
A52	881.82	Alkene	Morpholine	4.04
A56	867.80	Alkene	Morpholine	3.51
A57	901.85	Alkene	Morpholine	5.34
FS-3/84	789.68	Alkene	Amine	1.71
FS-3/85	771.67	Alkene	Methylated morpholine	2.59
FS-4/60	752.78	Alkene	Morpholine	4.5
FS-4/69	854.75	Amide	Morpholine	2.12
MGB-1	610.70	Alkene	Morpholine	3.81
MGB-1-Me	738.75	Alkene	Methylated morpholine	-0.42
MGB-1-NO	626.70	Alkene	N-oxide morpholine	2.62
MGB-2	611.69	Alkene	Morpholine	2.83
MGB-2-Me	853.76	Alkene	Methylated morpholine	-1.41
MGB-2-NO	741.71	Alkene	N-oxide morpholine	1.63
MGB-3	631.72	Alkene	Morpholine	4.13
MGB-3-Me	773.66	Alkene	Methylated morpholine	4.04
MGB-3-NO	761.75	Alkene	N-oxide morpholine	2.93
T/14	532.51	Alkene	Dimethylhydrazine	3.92
T/15	602.60	Alkene	Morpholine	4.06
T/23	707.62	Alkene	Piperazine	4.56
T/28	656.67	Amine	Morpholine	0.93
T/29	534.55	Amine	Morpholine	1.27
T/50	737.64	Alkene	Morpholine	4.37
T4/19	572.57	Alkene	Piperazine	4.24
T4/20	573.56	Alkene	Piperazine	3.26

8.2.3 The *in vivo* efficacy of MGB-3-NO against *L. donovani*

One of the most active of the compounds, MGB-3-NO was assessed for its *in vivo* activity against *L. donovani* using a murine model. In parallel, MGB-3-NO was also encapsulated in non-ionic surfactant vesicles (NIVs) to test if delivery and efficacy of MGB-3-NO could be enhanced using a drug delivery system. Mice were treated intravenously on day 7 post infection with PBS as a control, Empty-NIVs (150 mM lipid, 2 % v/v DMSO), MGB solution (5 mg/ml MGB-3-NO, 2 % v/v DMSO), MGB-NIVs (5 mg/ml MGB-3-NO, 150 mM lipid, 2 % v/v DMSO) or MGB-NIVs (1 mg/ml MGB-3-NO, 30 mM lipid, 2 % v/v DMSO). Neither MGB-3-NO in solution nor MGB-3-NO encapsulated in 150 mM lipid NIVs had a significant effect on the parasite burden in the spleen, liver or bone marrow of infected mice compared to control PBS (Figure 8.4). The diluted MGB-3-NO-NIV formulation (30 mM lipid) did however cause a significant ($p > 0.05$) reduction in the parasite burden in the livers of infected mice (Figure 8.4). Treatment using NIV formulations at the higher lipid concentration (150 mM), MGB-3-NO-NIV and empty NIVs produced a significant increase in spleen weight by the end of the experiment (Figure 8.4B).



	Organ Weight (g ± SD)	
	Spleen	**Liver**
Control	0.20 ± 0.01	1.35 ± 0.05
Empty NIVs (150mM)	0.36 ± 0.03**	1.73 ± 0.15
MGB	0.22 ± 0.01	1.29 ± 0.04
MGB NIVs (150mM)	0.48 ± 0.04**	1.74 ± 0.09
MGB NIVs (30 mM)	0.23 ± 0.02	1.44 ± 0.08

Figure 8.5. A) Parasite burden of mice infected with LV82 after 14 days of treatment with control (2% v/v DMSO), Empty-NIVs (150 mM lipid, 2 % v/v DMSO), MGB solution (5 mg/ml MGB-3-NO, 2 % v/v DMSO), MGB-NIVs (5 mg/ml MGB-3-NO, 150 mM lipid, 2 % v/v DMSO) or MGB-NIVs (1 mg/ml MGB-3-NO, 30 mM lipid, 0.4 % v/v DMSO). B) Table of organ weight of mice from the same experiment. Mice were infected with LV82 on day 0, treated on day 7 and sacrificed on day 14. (* $p \leq 0.05$, 40.20 ± 16.26 % reduction compared to control; ** $p \leq 0.01$; n=5).

8.3 Discussion

This work analysed the potential of a library of MGBs synthesised at the University of Strathclyde for the treatment of VL using luciferase expressing parasites. Correlation between the amount of BLI emitted and the number of promastigotes was excellent and shown to be stable over a prolonged period of time. Moreover, insertion of the luciferase gene in to the parasite did not appear to alter the susceptibility of luc amastigotes to common anti-leishmanial compounds or growth of either promastigotes or amastigotes *in vitro*. This would indicate that integration of the gene was not detrimental to parasites and they would be suitably analogous to WT for comparison of the activity of the MGBs.

Twenty nine MGBs were screened for their activity against *L. donovani* luc intracellular amastigotes. A universal molarity of 12.5 μM MGB was used to allow comparison of activity across the screen. The library contained the three lead MGB compounds, MGB-1, MGB-2, MGB-3 as well as a number of derivative compounds that were either methylated, had n-oxide morpholine residues, were truncated or had alterations to their tail groups. Strikingly, 5 of the 8 truncated MGBs showed no activity against *L. donovani* while one, T4/50 had the highest suppression of all the MGBs tested in the initial 29 compound screen. Early results from testing of the same library against *L. major* and *L. mexicana* indicate there are strain specific responses towards MGB (data not shown). *L. donovani* and *L. major* show similar susceptibility to the majority of the compounds screened whereas *L. mexicana* appears to vary significantly in that several of the MGBs that were active on *L. donovani* and *L. major* showed no activity against *L. mexicana* at all (unpublished

data). This is not entirely surprising given that the different species of *Leishmania* cause distinctly different diseases and have markedly separate loci of disease burdens globally. It does however highlight that a successful candidate drug may only be effective against the one species of *Leishmania* and that rigorous testing against multiple species would be required.

Fifteen of the 29 compounds were further analysed by determining more specific IC_{50} values against *L. donovani in vitro*. Of the compounds assayed, MGB-2-ME, MGB-3-NO and T4-50 were the most potent against *L. donovani* intracellular amastigotes and were as effective as the current anti-leishmanials PMM and MIL. MGB-3-NO was chosen for further investigation *in vivo* based on it having the lowest IC_{50}, its lipophilicity (log $D_{7.4}$/Log D) and the fact that T4-50 exhibited poor solubility at high concentrations. The lipophilicity of a drug can influence several parameters important in drug design such as; solubility, permeability, selectivity and clearance from the liver. The Log D value is an indicator of lipophilicity, with the greater the Log D, the more lipophilic the drug. As a rule of thumb, the optimal lipophilicity of a drug should fall between 1 – 3 Log D (Waring, 2010). Although MGB T4/50 showed a relatively high activity, the Log D of 4.37 was considered too high when compared to MGB-3-NO which had a lower IC_{50} and more suitable Log D of 2.93. A number of the compounds tested in this library have also been assayed for activity against both human and livestock trypanosomiasis and have shown similar efficacy against the different parasites (Scott *et al.* 2016). In particular, 5 compounds displayed IC_{50}s in the sub 50 nM range and of these 5, two that were screened in this study were among the most potent against *L. donovani*. Although

these data are at an early stage, they are none the less encouraging as it suggests that this MGB library may have a broad spectrum of activity against multiple protozoan targets. This is perhaps unsurprising as this particular set of MGBs are known to bind with high affinity to A-T rich regions of DNA, making the mini-circle DNA of the kinetoplasts of *Leishmania* and *Trypanosoma* parasites a likely target (Scott *et al.* 2016). Importantly however, Scott *et al.* note that the terminal amidine group that is crucial in the activity and uptake of diamidine is absent from this set of MGBs, therefore making cross resistance with amidine-resistant parasites unlikely.

MGB-3-NO was encapsulated into NIVs by Mireia Puig to produce MGB-NIVs. Freeze dried empty NIVs were rehydrated using MGB-3-NO dissolved in DMSO and PBS (total DMSO 2% v/v). Precipitation of the MGB solution was observed immediately after the addition of PBS, indicating that solubility in aqueous solution was poor. Final encapsulation efficiency of the MGB-NIVs formulation was greater than 30% (data not available, personal communication from Dr Mireia Puig). Moreover, vesicle size of MGB-NIVs was measured in the micrometre range which is generally considered large for a vesicular delivery system. Improvements to both the entrapment efficiency and solubility would be beneficial for further studies using an MGB-NIV formulation. Stability of the studies were not undertaken as MGB-NIV formulations were used immediately after encapsulation. In parallel to this study, Puig (2015) also investigated the addition of a cryoprotectant to improve the size of vesicles in formulations. With respect to the solubility concerns, cyclodextrins have the potential to improve solubility and delivery. Cyclodextrins are composed of

multiple rings of sugar molecules that are joined in a ring and are frequently used to solubilise AmB.

When tested *in vivo* using a mouse model MGB-3-NO in its free form did not hamper the proliferation of *L. donovani*, nor did the MGB-NIVs formulation at the higher concentration of 150 mM lipid. Only the diluted form of MGB-NIVs (30 mM) significantly reduced the parasite burden within the livers of infected mice. Even so, a parasite burden in the diluted MGB-NIVs treatment group was significant and did not lead to complete cure. Improving the entrapment efficiency and solubility of the MGB-NIVs formulation may be a method to improve efficacy of the MGB. Spleen and bone marrow burdens did not differ significantly between any of the treatments. The higher concentration MGB-NIVs formulation was associated with an increase in spleen weight in mice. It is possible that this is as a result of the low activity of the formulation allowing proliferation of the parasite. Splenic infections do not manifest until after day 28 therefore the activity of MGBs on the burden of parasites in this organ could not be assessed in this study. None of the treatments used were observed to be toxic to mice used and no reduction in body weights were observed.

MGBs are a very large and varied class of compounds, characterised by their ability to bind within the minor groove of DNA. Pentamidine, a well-known MGB can be used to treat CL and has been used to trypanosomiasis since the 1930s. New classes of MGBs to treat *Leishmania* are being sought and are showing promise, such as the arylimidamides (Stephens *et al.* 2003, Collar *et al.* 2011, Wang *et al.* 2011, Zhu *et al.*

2012). Early work identified several compounds with excellent potency against *L, donovani in vitro* that had activity in the sub 1 µM IC_{50} range (Stephens *et al*. 2003). Initial *in vivo* work was also encouraging and up to 90% reduction in liver parasitemia in murine models was obtained (Collar *et al.*2011). More recent testing on compounds with improved solubility however appear to show both a reduction in overall efficacy and an increase in notable toxicity of mice treated with the drug (Zhu *et al.* 2012). Studies in to the efficacy of arylimidamides by the Werbovetz group are on-going and are continuing to show potency against *L. donovani* (Pandharkar *et al.* 2014, Zhu *et al.* 2016). This particular class of MGBs appear to show significant activity against *Leishmania* that is in the sub micro molar range and arguably more active than the MGBs screened in this study. Similar to this study however, it should be noted that high activity *in vitro* does not necessarily translate in to comparable activity *in vivo* and toxicity must also be considered. A balance between activity and toxicity to the host must be achieved. Although early in the development process, several of the MGBs we have screened show acceptable activity against *L. donovani*. Furthermore, MGB-3-NO tested *in vivo* had no issues with toxicity against mice and the same is true for other compounds of this library that have undergone toxicity studies in different animal models (personal communication from Dr Fraser Scott, unpublished data).

Despite the fact that MGB-3-NO did not display the *in vivo* activity that one would have hoped for, several other compounds in the MGB library showed comparable *in vitro* results and it would be worthwhile profiling their activity *in vivo*. Moreover, the fact that several of the compounds tested showed comparable IC_{50}s to MIL and

PMM is encouraging as it vindicates the possibility that several compounds in the library have the potential to go forward for increased scrutiny. Caution should be urged however as this data is obviously from early *in vitro* screening and efficacy here may not necessarily translate *in vivo* as was reported here with MGB-3-NO. The fact that the promising compounds were not quite as active as AmB is not too discouraging however as although the aim would be to have as potent a compound as possible, easily administered and cost effectiveness are important considerations for anti-leishmanials and balance between all of these factors is needed. If any of these MGBs show broad spectrum against a range of target organisms, can be easily administered and have potency on par with MIL or PMM then they have the potential to become viable options in the fight against VL.

In conclusion, a number of MGBs appear to be promising in their activity against *L. donovani*. Three of the original 29 compounds have *in vitro* efficacy on par with two current anti-leishmanials, PMM and MIL. One of these, MGB-3-NO was taken forward and tested *in vivo* in a murine model. The free form of MGB-3-NO was not capable of supressing parasites *in vivo* but efficacy using and NIV formulation was capable of reducing the parasite burden in the livers of infected mice. Improvements to the formulation, including entrapment efficiency and vesicle size have the potential to improve this efficacy and should be investigated. So too should the *in vivo* efficacy of MGB-2-ME and T4-50. Interestingly, T4-50 was the only one of the truncated MGBs to show any activity towards the parasite *in vitro.* Furthermore, the compound is a natural fluorophore and so studies investigating the localisation of the compound within the parasite could potentially be done

using fluorescence microscopy. Work to characterise this library of MGBs for activity against not only *Leishmania* but a host of other parasites and bacteria is ongoing at Strathclyde and it will be interesting to follow the results in the hope of a potential new drug to treat VL.

The main outcomes identified in this chapter were:

- Twenty nine MGB compounds were screened for their efficacy against *L. donovani* intracellular amastigotes *in vitro*.
- Fifteen of the original 29 displayed satisfactory (>75%) killing of *L. donovani* in initial drug testing and were further characterised for their IC_{50} *in vitro* against the parasite.
- One of the lead candidates, MGB-3-NO, was put forward for testing *in vivo* in a BALB/c mouse model using free drug and two formulations of MGB encapsulated in NIVs.
- Both free drug and the higher concentration of MGB-NIV had no significant impact on the survival of *L. donovani in vivo* and although the lower concentration of MGB-NIV displayed parasite killing, it did not lead to complete cure.

Chapter 9. Further Work

Visceral leishmaniasis is a significant health problem in Northern India and Nepal where 90% of the 400,000 annual recorded cases occur. Chemotherapy to treat VL is limited and drug resistance in the parasite population is a threat to its clinical efficacy. Thus, understanding the basis of acquired drug resistance has the potential to identify protocols to extend the clinical life of available drugs is vital. In this study we employed a metabolomics approach in conjunction with genomic sequencing of clinical isolates from Nepal with different background susceptibilities to Sb to assess the impact of acquired resistance to PMM or MIL had upon these parasites. We saw this as imperative as Sb drugs have been used to treat VL for almost a century and therefore the mechanism(s) responsible for drug resistance to other chemotherapy may depend on the inherent Sb susceptibility of field strains. Here, resistance to MIL or PMM was selected for in *L. donovani* promastigotes with varied, inherent Sb susceptibilities due to the fact that *L. donovani* is the main species responsible for causing VL and these drugs are two alternatives to Sb currently used to treat the parasite. Sb resistance is associated with increased tolerance to macrophage microbicidal products (e.g. ROS, RNS) so it is possible that this could play a role in the development of resistance to other drugs (Carter et al., 2005, Vanaerschot et al., 2014). For this reason, PMM-R and MIL-R clones were evaluated for their "fitness" to infect macrophages and their response to macrophage killing mechanisms.

This study demonstrated that promastigotes of *L. donovani* could be easily selected *in vitro* for resistance to 74 μM and 97 μM of MIL and PMM respectively. Drug

resistant populations of parasites were subsequently cloned using the hanging drop method to produce clonal populations of parasites. Cloned parasites demonstrated variable degrees of drug resistance compared to the parental populations they were cloned from with some clones displaying higher IC_{50} values than their parents and others lower values. This confirms that despite being tolerant to the same concentration of drug, the parent population of drug resistant parasites contained a heterogeneous mix of parasites with variable susceptibilities to the selection drug. Resistance in the promastigote stage appeared stable as tolerance to both MIL and PMM was maintained even after removal of drug pressure in culture. Drug resistance in the promastigote stage was conferred to amastigotes and moreover, this was maintained even in the absence of constant drug pressure in the promastigotes. Our PMM-R promastigotes were cultured to a maximum of 97 µM PMM as beyond this, parasite growth was stunted. Two other published studies cultured parasites to this concentration as they were part of the same EC collaboration this study fell in to (Hendrickx et al., 2014, Bhandari et al., 2014). Interestingly, Hendrickx *et al.* selected for PMM tolerance in intracellular amastigotes in parallel to promastigotes. Here they cultured amastigotes that were tolerant to up to 500 µM PMM that conferred resistance to almost 200 µM in promastigotes. This is potentially very important as it emphasises the apparent differences that can be achieved depending on the stage used in the development of tolerance. Selecting resistance in the amastigotes was something that was targeted in this study and the process was initiated but sadly due to time constraints, it was not possible to complete. If this study was extended then

comparing the metabolomes of drug resistant parasites selected for in each stage of the parasite lifecycle would certainly be a priority. For example, the LdMT gene has been universally implicated in acquired resistance to MIL but all of these studies have focused on resistance selected in the promastigotes (Perez-Victoria et al., 2003a, Perez-Victoria et al., 2006, Dorlo et al., 2012). It is perhaps time that selecting resistance in *Leishmania* using intracellular amastigotes becomes the gold standard. Although it is more resource and labour-intensive, it would be advisable given that there does appear to be differences with promastigote selected resistance and the fact that amastigote derived resistance would be what would occur in endemic populations. In terms of hospital screening for resistance, currently, parasites are isolated from patients, grown in the absence of drug pressure and then assessed for their susceptibility to a particular drug. Although promastigote resistance appears to be a good marker for increased resistance in amastigotes, our study shows that you cannot tell specifically the level of resistance in the amastigote just from screening the promastigote. We would therefore recommend that screening for drug resistance focus on the amastigote stage to give a precise narrative of drug tolerance. We also think it would be beneficial to culture parasites isolated from patients in the drug to be screened immediately after isolation. While the parasites cloned in this study maintained their resistance in the absence of drug pressure and no loss of resistance has been reported in PMM-R parasites, data from MIL resistant *L. major* demonstrated that tolerance was lost when drug pressure was relaxed (Turner *et al*., 2015). Although it is only speculation, this could be an explanation of why MIL resistant parasites have not

been isolated from any patients who have relapsed after treatment with MIL (Rijal *et al.*, 2013). While it would make growing isolated promastigotes more difficult, it would perhaps provide a more accurate screening process to assess the resistance profile of WT populations. Another reason for the apparent inability to culture MIL resistant isolates from relapse patients could be that, as reported by Hendrickx *et al.* resistance to MIL in the patients' amastigotes may not be translated in to promastigote and therefore not appear resistant to the drug when tested (Hendrickx et al., 2014).

An alternative way to screen for resistant parasites could be found in analysing the metabolome or lipidome of isolated parasites. Ideally, we wished to identify a marker common to all parasites resistant to the same drug that would act as an indicator of resistance. The vast majority of differences between WT and MIL-R clones were found in the lipidome of parasites. This is not particularly surprising given that MIL itself is a phospholipid. The disruption of the LdMT gene would likely affect the uptake of several other PLs and similarly, adaptations to cope with the action of MIL would likely have knock on effects to other similarly structures lipids. It is still unclear in this study whether the large scale alterations in the lipidome of MIL-R parasites is a mechanism that helps protect the parasites from the effects of a drug, or as a consequence of the resistance to MIL, or perhaps a combination of the two. There was however no consensus on lipidomic alterations in response to MIL selection between Sb-S MIL-R and Sb-R MIL-R. In fact, the two clones underwent considerable alterations of their lipid architecture but in distinctly different ways. It is however possible to postulate that although markedly different,

both sets of changes could lead to alterations to the lipid membrane that would change the fluidity of the membrane to decrease permeability. This is backed up in part with the observation that MIL uptake was significantly reduced in MIL-R clones. This reduced uptake however is also likely to be at least in part due to genetic adaptations associated with MIL selection, namely changed to the LdMT gene. This was one of the most significant genetic observations of this study, which has also been reported in other studies (Perez-Victoria et al., 2003a, Perez-Victoria et al., 2006). These changes included loss or significant disruption in the gene sequence for the LdMT gene leading to a failure to transcribe RNA and the chromosome copy number for the chromosome coding for the LdMT gene was reduced. This genetic change was reflected in the near absolute decrease of internalised MIL inside resistant parasites. Therefore developing of an assay to measure MIL uptake by *L. donovani* parasites would be a clear indication of its MIL-R phenotype. Our studies uncovered that resistant promastigotes accumulated between 10-20 times less MIL inside the cell compared to WT. Likewise, studies using radiolabelled MIL demonstrated a 15 fold difference in MIL-R promastigotes (Perez-Victoria *et al.*, 2003a). A simple screen assessing the quantity of MIL inside of promastigotes after incubation with a known concentration of MIL in the media could be utilised to screen for promastigotes with a tolerance to the drug. Our assay used here could be employed and adapted although it would require the use of LC-MS which can be prohibitively expensive. Alternative screens could perhaps be developed utilising fluorescently labelled MIL and screening using fluorescent microscopy for localisation of MIL within promastigotes could be applicable. This sort of assay

however would be hard to quantify. Perhaps an easier and more routinely available assay could be to probe for the LdMT gene and its transcription. This gene is heavily implicated in MIL resistance and it is likely that it is the main culprit responsible for excluding MIL from MIL-R parasites. We would however advocate probing for transcription products of the LdMT gene as one of our MIL-R clones had gDNA encoding LdMT but lacked any detectable transcripts of the gene. As explained in the discussion of Chapter 5, although it was unexpected that the presence of gDNA could lead to a lack of a transcript, there is some evidence in similar studies on African trypanosomes that suggest it is possible (Stewart et al., 2010).

Development of PMM resistance appeared to follow a more predictable pattern than MIL resistance. The majority of adaptations associated with selection of PMM-R promastigotes concerned metabolites rather than lipids. This is in contrast to selection of MIL-R promastigotes in which the majority of alterations were found in the lipidome of resistant parasites. Moreover, PMM resistance in all three clones tested had either common metabolite alterations or changes to pathways associated with resistance to oxidative stress, specifically proline and related metabolites. This suggests that PMM resistance, at least in the promastigote stage, is correlated with a particular and perhaps predictable mechanism. A commonality in development of resistance would be useful in two ways; One, it would allow development of a biomarker assay to detect strains in patients that are already resistant to PMM and help choose the correct drug to treat VL. Two, if the route at which parasites develop resistance is predictable then it could be possible to

develop strategies that could delay or prevent the onset of resistance or counteract the development of acquired resistance by co-administering another drug that could work in tandem with PMM.

Profiling the fitness of MIL-R and PMM-R clones also revealed differences in the consequences of drug resistance between selection of MIL and PMM. The development of MIL resistance appeared to be overall detrimental to promastigotes in either their ability to infect macrophages, influence the macrophage cytokine production, or resist oxidative stress. If MIL does consistently lead to a loss of fitness then it is somewhat good news for the future of MIL treatment. It has long been postulated that MIL resistance in the wild is only a matter of time but as yet, no definitively MIL resistant parasites have been isolated from relapse patients. In contrast, PMM-R clones showed increased resistance to the aforementioned criteria of fitness. In addition, the inherent PMM-R resistant Sb-S WT appeared more resistant to oxidative stress compared to Sb-R WT. This backs up previously published data that correlates an increase in tolerance of macrophage induced oxidative stress with tolerance of PMM (Bhandari et al., 2014, García-Hernández et al., 2015). Given these data, it would be reasonable to tentatively postulate that in the wild, resistance to PMM is far more likely to develop that MIL as PMM-R parasites appear fitter than those resistant to MIL. It would also explain why we were able to isolate the Sb-S WT strain from an area where Sb use is obsolete due to widespread Sb resistance. The inherent resistance to PMM which we uncovered could well be giving the Sb-S WT a selective advantage over other populations. With

SSG use now abandoned in the region, the selective pressure which it was susceptible to is now longer acting to suppress the population yet resistant parasites persist.

In the future, studies could focus on a number of areas. Studies using metabolomic profiling of drug resistant parasites would be an interesting and worthwhile venture with several avenues to explore. Firstly, this study only explored MIL and PMM adaptation in two and three clones respectively. These sample sizes obviously need to be expanded to make accurate assumptions on patterns of the mechanisms involved in resistance to either drug. Profiling isolates from relapse patients would also be an interesting avenue which would possible shed light on the mechanisms or reasons behind the relapse and elucidate populations of parasites that may have developed a tolerance to treatment. The two MIL-R clones profiled in this study altered their metabolomes in distinctly different ways yet both were resistant to significant concentrations of MIL. It is still debatable whether the changes in the lipidome of MIL-R parasites were beneficial as a mechanism of resistance or a consequence of another mechanism, for example the loss of the LdMT transporter. It is entirely likely that disruption of the LdMT also compromises the accumulation and biosynthesis of lipids in MIL-R promastigotes. Lipid biosynthesis in *L. donovani* is not fully understood and extrapolation of knowledge from *T. brucei* is often used. The basic framework of lipid metabolism in *L. donovani* is understood but there are gaps that could yet be filled in. This could be achieved by profiling the metabolome of a number of different *L. donovani* strains with particular attention given to the

lipidome to give a clearer picture of the entirety of the lipidome. More focused studies could then target particular pathways or subsets of lipids and use radiolabelled isotopes or fragmentation to elucidate more accurately pathways and the structure of particular metabolites or lipids (Storm et al., 2014, Welti et al., 2007, Li et al., 2013, Postle and Hunt, 2009, Botte *et al.*, 2013). Studies focusing on the acquisition and synthesis of PLs in *L. donovani* would be valuable. One method to achieve this could be found using radio isotope labelled tracing experiments. Labelling either the glucose, ethanolamine or PL source in culture media and analysing what metabolites the isotopes are incorporated in to would likely reveal a great deal about lipid biosynthesis in the parasite. Such studies could perhaps also be used to confirm the mechanism of headgroup switching of PCs to supplement the PE pool in Sb-R MIL-R which was observed in this study.

Another important but complex task would be developing a method to accurately analyse the metabolome of intracellular amastigotes. This would be challenging in firstly, harvesting sufficient numbers of amastigotes to accurately capture a picture of the metabolome. Secondly, distinguishing between macrophage and parasite metabolites would be tricky and would need robust development. Achieving this however would open up metabolomics with *Leishmania* to a more accurate and clinically relevant phase. From the genomic sequencing, we can say with certainty that the LdMT transporter is rendered useless in both MIL-R clones and it also seems likely that it is playing at least a small role in the alteration of lipidomes of MIL-R promastigotes. What the consequences are in the amastigote stage however

is not known for certain. SSG for example, is only effective against amastigotes, highlighting that stage specific drug effects exist. Currently however, using promastigotes in metabolomic analyses is the most feasible option.

Our study, along with several others suggest that while MIL resistance is associated with a decreased uptake of MIL inside the parasite and is easily acquired *in vitro*, a fitness consequence appears to result that lessens both the infectivity of the parasite and its tolerance to macrophage killing mechanisms. On the other hand, PMM resistance results in parasites that have an increased capacity to tolerate the effects of macrophage killing effectors. This is good news for the future of MIL treatment as it suggests that the development of resistance to MIL in endemic regions is not as imminent as first predicted. Conversely, this study predicts that not only are there some PMM resistant populations already found in the field but that the development of PMM resistance could likely lead to parasites that are better suited to outcompete their non-PMM resistant populations. Focus should perhaps move more towards studying the mechanisms of PMM resistance in an effort to better understand and perhaps control the problem. Although PMM is not used as routinely as MIL or L-AmB, its cheap cost and efficacy, particularly in combination therapy means it would be an important weapon in the arsenal against *L. donovani*. Indeed combination therapy is perhaps now considered the best option for future treatment of leishmaniasis, particularly in patients co-infected with HIV (Mahajan et al., 2015, van Griensven et al., 2010, Olliaro, 2010).

To conclude, Resistance to MIL may not occur as readily as previously thought and this could be due to a number of factors such a decrease in fitness of parasites that have acquired resistance to the drug. The results from profiling the metabolome of MIL-R parasites revealed that the mechanisms and consequences of acquiring resistance to MIL are not predictable but is largely centred on lipid metabolism, a likely consequence of loss of the LdMT. In contrast, acquired resistance to PMM would appear to follow a more predictable pattern, with increased levels in several metabolites associated with increased tolerance to oxidative stress measured in all our PMM-R parasites. Furthermore, PMM resistance was associated with an increased fitness of parasites in response to several macrophage killing mechanisms compared to WT. This suggests that if they were to emerge, parasites resistant to PMM may have the capability to outcompete populations of no parasites susceptible to the drug, even without the selective pressure of PMM.

Chapter 10. References

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Appendix

Publications

C D Shaw, J Lonchamp, T Downing, H Imamura, T M Freeman, J A Cotton, M Sanders, G Blackburn, J C Dujardin, S Rijal, B Khanal, C J Illingworth, G H Coombs, K C Carter. In vitro selection of miltefosine resistance in promastigotes of Leishmania donovani from Nepal: genomic and metabolomic characterization. *Mol Microbiol*. **2016**, 99(6): 1134-48.

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